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EDITED BY

L. D. DAKIN, New York City.      LAFAYETTE B. MENDEL, New Haven, Conn.  
L. K. DUNHAM, New York City.      A. N. RICHARDS, Philadelphia, Pa.

WITH THE COLLABORATION OF

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R. H. CHITTENDEN, New Haven, Conn.  
OTTO FOLIN, Boston, Mass.  
WILLIAM J. GIES, New York.  
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V. C. VAUGHAN, Ann Arbor, Mich.  
ALFRED J. WAKEMAN, New Haven, Conn.

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PROCEEDINGS OF THE AMERICAN SOCIETY OF  
BIOLOGICAL CHEMISTS.

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EIGHTH ANNUAL MEETING.

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# PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

## PRESIDENTIAL ADDRESS.

### THE PHYSICS OF SECRETION AND EXCRETION.

By A. B. MACALLUM.

On the last occasion I addressed the Society on the question of the source and causation of the energy of muscle contraction. To-day I desire to discuss a kindred subject: the physical forces that determine secretion and excretion.

In selecting this topic I am well aware of the difficulties it offers to any one who wishes to become seized of an approximate concept of all the forces that are involved in such processes. Some of these forces are obscure and some of them are too elusive to be brought forward to reveal the part they play. It is, however, worth our while to attempt on an occasion such as the present to review our knowledge of this subject, to take stock of it as it were, in order that we may know how far our achievements in it avail to explain its problems in a rational or philosophical manner. It is indeed only in this way that we hope to avoid a wrong path or following a false light.

The difficulties leap to the eye when one examines the subject. Take in illustration a unicellular form like an amoeba. It is a minute mass of protoplasm with a nucleus which, though apparently not so simple, is of a structure and composition that has in a measure been revealed and explained. Its simplicity is such that the average observer wonders how such a minute mass could manifest the properties it possesses. It moves, it ingests food material and digests it, it absorbs and assimilates the products, it takes up oxygen and gives off carbon dioxide, it grows in volume and divides and new cells thus formed are ever ready under appropriate conditions to go through the same rôle till the end of eternity. If on the other hand such a mass were to be treated with a toxic agent, or if it were deprived for a time of oxygen, these manifes-

tations would cease once for all and life would go beyond recall. Yet the minute mass appears at the moment it dies just as it did during life. There may be not the slightest difference observable in the general constitution. Only the manifestations are lacking and we say that it is no longer living.

The average observer, let me say again, viewing the living mass and then the dead is prone to think that the difference between the two does not reside in any physical distinction, that the force or forces involved in these manifestations cannot be of the type which we term physical or chemical. It is difficult for him to believe that there is any difference, chemical or physical, between the living and the dead, and yet there is a vast difference between them in the manifestations which are observable in one, and which are wholly absent from the other. This difference, he is prone to claim, constitutes a gulf which cannot be bridged by any intellectual structure built out of the physical and chemical lore that we possess, or that we shall possess.

It is not only the ordinary observer who takes this point of view. There are not a few chemists, physicists and biologists who take it also and in consequence we have a school of thinkers who postulate that fundamentally there can be no explanation of life in terms of physical or chemical laws and that there is a science of life or of living matter distinct, fundamentally different, from either or both of the sciences, physics and chemistry. They hold that there resides in, and operates through living matter a force or entity which does not obey physical laws but which can direct the operation of physical and chemical laws in living matter itself. What this "force" is no one has defined and what becomes of it when the structure through which it operates dies no one can tell us, or has deigned to tell us. Nor are we informed how we are going to constitute a science comprehending the operation of such an elusive force.

Now it may be freely confessed that this attitude makes a strong appeal to certain tendencies of the human mind. One of these involves the attraction that mysticism presents. This is a tendency as old as the human mind, with a history that extends back to the very beginning, millions of years ago, of the human race. In the remote past the part played by mysticism was a crude one, but the rôle it performed became more and more refined and in

is, their removal from the cells to the lumen is not the result of osmotic pressure. The difficulty of accounting for these processes were supposed to be met when the van't Hoff-Arrhenius explanation of osmosis was advanced but that can no longer be maintained. To explain thus the secretion in this case would demand that before secretion begins in the submaxillary gland the lumina of the acini are filled with a concentrated solution of electrolytes or non-electrolytes which cannot diffuse into the cells, and, in consequence, fluid would pass from the latter into the lumen. Such a concentrated solution there might be at the very moment secretion begins but it could not obtain when secretion is prolonged and the flow through the cells of the secreted fluid must be due to some other force.

In the kidney the greater part of the water of the excretion is derived by filtration through the glomeruli from the blood. The constituents of the urine other than water are eliminated from the blood through the convoluted tubules and Henle's loops. If now the urine were merely the product of diffusion and filtration alone its osmotic pressure would never exceed that of the blood plasma. The concentration, in urea and salts, of the urine is ordinarily such that its osmotic pressure greatly exceeds that of the solutes in the blood plasma and lymph. Here, manifestly, work has been done by the cells of the tubules for the solutes have been driven through the cells in the direction of their greater concentration, that is, in the direction of greater resistance.

We have then, in the salivary glands and in the kidney a form of activity which is opposed to the operation of the physical processes of diffusion, filtration and osmosis. If the cells concerned in this activity are systems which are governed by physical and chemical laws the activity itself involves a negation of the second law of thermodynamics, which postulates that energy, *e.g.*, heat, always passes from a higher or more available form to a lower or less available state and that only under such a condition can work be done. The production of a high manometric pressure in the saliva in the duct of a salivary gland, or the development of a high osmotic pressure in the urine, involves a passage of energy from a lower or less available form to a higher or more available one. To account for this result we may deny the validity of the second law or postulate the existence in each cell of an entity which can, on occasions neutralize the operation of the second law.

Helmholtz doubted whether the second law does apply in living matter and Boltzmann also was of the opinion that there might be conditions in the physical world under which the second law may fail to act. A more fundamental objection may be cited. That the second law cannot be of universal application would seem to follow from the fact that after the lapse of æons energy is not uniformly distributed throughout the visible universe. For this and other reasons it is open to question whether the generalization involved can be accorded the status of a law. In our world of experience, however, it does operate as a law and when we find an apparent negation of it, as illustrated in salivary secretion and renal excretion we must endeavor to explain the fact in accordance with the law or determine whether the negation is caused by the conditions postulated by Helmholtz and Boltzmann.

It is of interest here to point out that J. Clerk Maxwell conceived that the second law could be set aside under special conditions. In illustration of these he supposes the existence of a being, or demon as it were, whose faculties are so sharpened that he can follow every molecule in its course and who can accordingly discriminate between the fast-moving and the slow-moving molecules in a gas. He also supposed a vessel filled with gas and divided into two portions A and B by a septum in which there is a small hole which can be closed or opened, by a shutter as it were, and that the "demon" opens or closes this hole so as to allow the swifter molecules to pass from A to B and the slower to pass from B to A. After a time the temperature of B will have risen while that of A will have fallen, that is, more energy has flowed into B and at the expense of that which was originally in A and in contradiction to the second law.

There would, we may suppose, be the same or a like contradiction if the demon used part of the energy in A to move the shutter when the aperture in the septum is to be closed or made patent. The quantity of energy so used need not be stipulated and it may be a considerable portion of that in A but so long as the quantity of energy in B is greater than it was before the shutter is brought into use the result implies a violation of the second law.

The parallel between the "demon" engine so modified and the cells of the salivary gland or of the convoluted renal tubules is a close one, for not only is energy in these cases concentrated rather

than diffused uniformly, but also the concentration is accomplished at the expenditure of some energy. Barcroft has established that when the salivary glands are secreting they absorb more oxygen than they do while in the inactive stage and Brodie and Barcroft have shown that in the dog's kidney during diuresis the oxygen absorbed is very great. If the oxygen so absorbed is used up on the oxidation of a protein they calculated that only one two hundred and twentieth of the energy thus liberated is expended in the concentration of the urine. That there had been this large amount of energy liberated was indicated by the fact that a quantity of carbon dioxide almost exactly equal to the amount of oxygen absorbed, was set free.

The parallel between the "demon" engine and the cells in question may be conceived to be a more complete one by the vitalist, who predicates that each of the cells acts as it does because in each resides a force, an organizing or directing entity, an entelechy as it has been called, which in some manner plays in the cells the part that is performed by the "demon" in Maxwell's mechanism or engine.

The difficulty of reconciling the facts of submaxillary secretion, and of renal excretion with the present acceptance of the second law of thermodynamics is not as great as would appear from this presentment of it. If it were it would constitute a fact in support of the doctrine of vitalism which, in our present knowledge of the physical forces that operate in the living cell, it would not be easy to pass over. As it is now, however, it is regarded as insuperable by all those who hold that we know all in that line which may be of any service and that such knowledge fails to explain the problem.

Is it, however, possible that the second law may be set aside in some other way than that postulated in Maxwell's "demon" engine? Can we imagine a mechanism like the latter but in which the demon is not a factor, and yet the results would be the same? If so, it would parallel so far as effect is concerned, the activity in either the salivary or the renal cells.

Such an engine is theoretically possible. It would involve a septum with shutters in the pores of the same which would open in one direction only, *e.g.*, from A to B. These shutters, further, should not swing open except when they would be hit by molecules in A which have in their movement a velocity higher than the average

velocity of the molecules in either B or A. When a single shutter would be struck on its two faces by two molecules it would remain closed if the impacts were the same or if the impact on the side of B were greater than on the side of A, but it would open if the impact on the side of A were greater than on the side of B and the molecule giving this impact would pass into B. After a time there would be in B not only a slightly larger amount of kinetic energy, but also a slightly larger average of energy to each molecule than in A.

Such a septum would, in its construction, postulate an ingenuity which is capable of application to ultramicroscopic service. Such an ingenuity is not inconceivable and, therefore, it is permissible for us to suppose that in some forms of living matter there is to be found a mechanism which simulates in its action the septum of an engine like Maxwell's, but without its "demon." We do not need to imagine that the mechanism is a septum with shutters opening in one direction only. All that we are required to postulate is a special association of structure with forces, physical and chemical, which are known to play a part in cell life, forces which in their operation in such a system result in the unequal distribution of energy that we find in the cases of secreting activity in the salivary and renal cells.

We must not suppose that the cell is a septum. The latter structure is homogeneous or, to put it in another way, is formed of but one phase or state of substance, whereas the cell, even the simplest, is constituted of unhomogeneous material in which the phases are not few but many and the differences between them may extend in gradation from one extreme to another, forming thus a series which, however, are not localized in this order throughout the cell.

When, therefore, the cell is examined it is found to present differences in its composition in its different parts. These differences are mainly evident in the differences in the structure, differences which the cytologist observes, but they are evident also to the microchemist who can at least in a number of cases demonstrate that substances or compounds are present in one part of the cytoplasm of the cell and not in another, and this alone would indicate that phases in the parts of the cell so concerned are of different physical and chemical characters.

than diffused uniformly, but also the concentration is accomplished at the expenditure of some energy. Barcroft has established that when the salivary glands are secreting they absorb more oxygen than they do while in the inactive stage and Brodie and Barcroft have shown that in the dog's kidney during diuresis the oxygen absorbed is very great. If the oxygen so absorbed is used up on the oxidation of a protein they calculated that only one two hundred and twentieth of the energy thus liberated is expended in the concentration of the urine. That there had been this large amount of energy liberated was indicated by the fact that a quantity of carbon dioxide almost exactly equal to the amount of oxygen absorbed, was set free.

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In secreting and excreting cells surface tension plays a special rôle. The significance of this is seen in the distribution of the salts in the cells and it is due to the action of the Gibbs-Thomson principle which is to the effect that when a substance lowers the surface tension it condenses in the surface, and when it raises the surface tension it is less abundant in the surface than elsewhere in the solution. It has been found that when a portion of the surface of an animal or vegetable cell has a lower tension than the remainder of the cell, salts, those of potassium for example, are condensed there and accordingly condensation in a portion of a surface of a cell is a clear indication that the surface tension there is low. Now, potassium salts are found condensed on the crinic surfaces of salivary and pancreatic cells and of the cells of the renal convoluted tubules and we may, therefore, infer with considerable degree of certainty that the crinic surface of a secreting or excreting cell has a lower surface tension than the trophic surface has. Low surface tension is therefore associated with lessened viscosity and the special phases of the crinic zone.

It is very probable that this inequality in the surface tension on the surface, not only of secreting and excreting cells, but also of those which extend and maintain pseudopodia or tentacles, as in the case of *Acineta*, is due to the differences in the character and distribution of the phases present. The potassium and other salts present in secreting and excreting cells may enter into special relations with some of these phases of the crinic zone and the combinations so effected may lower the surface tension of the portion only of surface immediately adjacent to that zone, with the result that the salt becomes condensed there. It is only in this way that we can explain such inequalities in the tension of the surface of a single cell for if the phases were the same everywhere in it the surface tension, whether high or low, ought to be the same all over its surface.

This condensation on the crinic surface must be, as I have elsewhere pointed out, a factor in bringing about the concentration of urine. It is generally accepted that the water of the urine is largely if not wholly separated by the glomeruli from the blood and that the constituents, organic and inorganic, are excreted by the tubules. Now in the case of potassium salts which, as a rule, are proportionately much more abundant in the urine than in the blood, the water

excreted by the glomeruli, passing down the lumina of the tubules, would dissolve the condensations in the crinic surfaces of their cells and thus before the collecting tubule is reached the concentration would far exceed that of the potassium in the blood. Similarly with the other solutes, probably, condensations of which in the crinic surfaces we cannot demonstrate simply because we have no microchemical methods to indicate their distribution.

That explains the excretion of urine of an osmotic pressure higher than that of the blood plasma or lymph. The question now is, how can we account for the manometric pressure in the saliva being greater than obtains in the blood supply of the salivary glands? Does the water condense on the crinic surfaces of the salivary cells in the same way that potassium does in them and in the renal cells? That under certain circumstances water molecules may be condensed on surfaces of a system where the tension may be lowered by it is not at all improbable, but under circumstances which are not likely to obtain in secreting and excreting cells, and we must accordingly turn elsewhere for an explanation.

I have already mentioned the force known as intrinsic pressure in liquids. This force is due to the attraction of the molecules for each other, and it is the same force that in the surface of liquids is known as surface tension. The magnitude of this force is great but it is difficult to estimate it exactly for reasons which are evident. Thomas Young in 1805, by a method which he did not describe, estimated the intrinsic pressure of water to be about 23,000 atmospheres, and Dupré (1868), determining it from the mechanical heat of steam, placed it at 25,000 atmospheres, while van der Waals, basing his estimate on the value of  $\frac{a}{v^2}$  in his equation

$$\left(p + \frac{a}{v^2}\right) (v - b) = RT$$

gives its value as ranging between 10,500 and 10,700 atmospheres. Intrinsic pressure is, therefore, an almost inconceivably great force.

Low surface tension, except when the surface molecules are affected by an electric charge, is due to a diminution of the attraction between the superficial molecules of the surface and this postulates a diminution of the attraction between the immediately or mediately underlying molecules. Where, therefore, as in the

crinic surface of a cell there is low surface tension there is an intrinsic pressure in the crinic zone less than that in the trophic zone. It may be suggested that the phases in the crinic zone are responsible for this lower intrinsic pressure which is less than that of water.

Now this difference in the intrinsic pressure between the two zones of the cell is not without its effect on the movement of fluid in the cell. Left to itself equilibrium would tend to establish itself as it does in the dead cell. In the living the difference is maintained from the manner in which the secreting or excreting cell is nourished and the direction from which the cell is nourished. The trophic side and the trophic zone accordingly, tend always to be different from the other poles of the cell. There is nevertheless always a tendency to approximation of the intrinsic pressures in the two zones just as there is a tendency for the energy in a closed system to become uniform but in the living active cell the result is never uniformity but difference.

The only way in which uniformity can be effected is through the flow into the crinic zone of water which, if sufficient, would result in approximating the two pressures. Of all liquids except, perhaps, mercury, water appears to have the highest intrinsic pressure and, therefore, the more of it there is in the crinic zone the higher the intrinsic pressure there. Water, accordingly, flows or diffuses into the crinic zone to make the intrinsic pressure the same as elsewhere in the cell. Here we have the operation of the principle of imbibition or "Quellung" of colloids. If a colloidal system is surrounded by water the latter will diffuse into the former and raise its intrinsic energy, if it is in contact with or surrounded by another colloidal system water will diffuse from one into the other and the intrinsic energy of both will be equalized. In the two colloidal systems in the secreting cell, the trophic and the crinic zone, a similar relation obtains and into the crinic zone passes water which would tend to accumulate there but it also flows or diffuses through the crinic surface into the lumen and thus during secretion the equalization of the two intrinsic pressures in the cell is never attained. Such a flow would account for the manometric pressure in salivary secretion which is evidently derived from but a minute fraction of the energy utilized in the active gland.

This accounts for the flow of water in all glands and even in the

urine of the rat, horse, cow, goat, racoon and badger. The results are summarized in the table below, in which we have included the data previously published by ourselves, as well as (designated by an asterisk) those furnished by other observers. In this table "purine" is to be understood as including allantoin, while "uricolytic index" indicates the ratio of allantoin nitrogen to the sum (taken as 100) of allantoin and uric acid nitrogen. Excepting the sheep and the coyote, which were starved, all our animals were maintained upon an adequate purine-free diet.

ORDER AND SPECIES	PER CENT OF TOTAL "PURINE" NITROGEN			URICOLYTIC INDEX	DAILY OUTPUT OF "PURINE" N IN MOMS. PER KILO
	Allantoin	Uric acid	Bases		
Marsupialia.					
Opossum.....	73	21	6	76	5
Rodentia.					
Rabbit.....	94*	6*		94*	31*
Guinea-pig.....	91	6	3	93	32
Rat.....	93	4	3	96	50
Ungulata.					
Sheep.....	71	14	16	84	4.5
	61	11	28	85	3.6
Goat.....	81	7	12	92	18
Cow.....	93	7	0.5	93	18
Horse.....	87	12	1	88	2.6
	79*	21*		79*	
Pig.....	89*	2*	9*	98*	
Carnivora.					
Raccoon.....	92	6	2	94	17
Badger.....	97	2	1	98	30
Coyote.....	96		4	99?	23
Dog.....	97*	2*	1*	98*	22*
Cat.....	97*	3*		97*	
Primates.					
Monkey.....	66	8	26	89	4.5
Chimpanzee.....	0*	100*		0*	
Man.....	2*	90*	8*	2*	2.5*

\* Figures furnished by other observers.

Many of the above results are only preliminary, and there are in the ratios, as they stand, several irregularities that call for further study. In the meantime the following conclusions may be pretty safely drawn. 1. In all species except man and the higher

apes (Wiechowski) allantoin is quantitatively by far the most important product of purine metabolism. 2. In carnivora and rodents "uricolysis" is always practically complete; in the monkey it is slightly, in the opossum decidedly, less so; among ungulates there is no definite rule. 3. While in the majority of species uric acid is excreted in greater abundance than purine bases, there are instances, especially frequent among the ungulates, where the reverse is true; the precise relation between the two is, even for the species, not so constant as the uricolytic index. 4. The total endogenous purine metabolism per unit of weight is in a general way inversely related to the size of the animal; but there are notable exceptions.

#### IMMUNIZATION AGAINST THE ANTICOAGULATING EFFECT OF HIRUDIN.<sup>1</sup>

By MIGUEL VERA AND LEO LOEB.

(From the Department of Pathology, Barnard Free Skin and  
Cancer Hospital, St. Louis.)

Intravenous injections of solutions of hirudin into mice on six or seven consecutive days causes the appearance of substances in the blood of the mice which counteract the effect of hirudin on the blood. These substances inactivate a certain quantity of hirudin *in vitro* and *in vivo* as far as the anticoagulating effect of hirudin is concerned. In a mixture of serum containing this substance and of hirudin, to which blood is added afterwards, the action of the hirudin was counteracted to a greater extent if the blood was added directly after the mixture of serum and hirudin had been prepared than in cases in which the mixture was allowed to stand for fifteen minutes at room temperature. We may therefore conclude that the antibody is not a ferment which gradually destroys the hirudin. The action of this substance is not altogether specific, inasmuch as it counteracts also the anti-coagulating effect of sodium fluoride. This substance is without action on blood which has been made incoagulable through inactivation of calcium, as after addition of oxalate, citrate and magnesium chloride to the blood. We may therefore conclude that sodium fluoride does not prevent the

<sup>1</sup> Read by title.

## SOME ANAPHYLACTIC REACTIONS.

BY H. C. BRADLEY AND W. D. SANSUM.

*(From the Department of Physiology, University of Wisconsin.)*

1. *Specificity of haemoglobins.* Haemoglobins of different origins were found to possess a high order of specificity. Guinea-pigs sensitized to crystallized dog haemoglobin, do not react to, or respond only mildly to subsequent injections of haemoglobin from cat, pig, calf, beef, horse, goat, sheep, man, turtle, pigeon, and chicken. Such pigs are highly sensitive however to subsequent injection of dog haemoglobin. Pigs sensitized to beef haemoglobin react to calf haemoglobin, but not to the others named above.

2. *Isogenous proteins.* Guinea-pigs injected with small amounts of guinea-pig tissue brei appear to be more severely intoxicated upon subsequent injection than normal animals. There are many individual exceptions, but in a considerable number of injections the reactions appear more acute and the prostration of longer duration regularly in the previously sensitized animals. A low order of specificity between different isogenous proteins seemed also indicated in the experiment.

## THE MODE OF ACTION OF SOY BEAN UREASE.

BY DONALD D. VAN SLYKE AND GLENN E. CULLEN.

*(From the Rockefeller Institute for Medical Research, New York.)*

The concentration of a urea solution can be varied between 0.2 per cent and 10 per cent without influencing the rate at which ammonia is formed from the urea by a given concentration of urease. If the concentration of enzyme is varied, however, the initial rate of urea decomposition varies proportionally. These facts indicate that the enzyme combines with a definite maximum of substrate(urea), and that the presence of amounts of the latter in excess of that required to bind all the enzyme is without influence on the rate of the reaction. The mass of reacting substance is not the total amount of substrate present, but that portion of it combined at the moment with enzyme.

As the reaction proceeds the ammonia formed causes a progressive retardation of the rate, until the latter has been depressed

to approximately one-eighth the original velocity. Beyond this point the rate remains nearly constant.

The activity of the enzyme is doubled by each 10° increase in temperature between 10° and 50°. The optimum lies at about 60°, but the increase above 50° is comparatively slight.

The quantitative relations have been expressed in a differential equation, which when integrated gives the formula:

$$t = \frac{4}{E \text{ antilog} \cdot 03 T} \left( x - b \log \frac{c+x}{c} \right)$$

( $t$  = time of reaction;  $E$  = enzyme concentration;  $T$  = temperature centigrade;  $x$  = concentration of ammonia formed;  $b$  and  $c$  are constants.)

### GLYCOL ALDEHYDE IN PHLORHIZINIZED DOGS.<sup>2</sup>

By R. T. WOODYATT.

(From the Otho S. A. Sprague Memorial Institute Laboratory of Clinical Research, Rush Medical College, Chicago.)

Glycol aldehyde prepared from dioxymaleic anhydride by Fenton's method administered in doses of 5, 6, and 10 grams to fully phlorhizinized dogs, caused a rise in the absolute quantity of sugar excreted, but also a corresponding rise in the nitrogen, the D : N ratio remaining substantially as before. Administration was subcutaneous and intravenous (three experiments).

### TRIKRESOL AS A SUBSTITUTE FOR TOLUENE IN ENZYME WORK.

By SARA S. GRAVES AND PHILIP ADOLPH KOBER.

(From the Harriman Research Laboratory, Roosevelt Hospital, New York.)

The disadvantages of toluene are: 1. It evaporates readily. 2. It often produces a cloudiness which interferes with optical methods. 3. It clings to glass in oily drops introducing errors in volumetric measurement. 4. Its bactericidal power is uncertain.

<sup>2</sup> Read by title.

ably affect the openness of the shell. Oysters when tightly closed take no oxygen from the surrounding water. Oysters show considerable resistance to lack of oxygen. The common clam *Mya Arenaria*, shows a higher oxygen requirement than the oyster, but, in proportion to its dried weight is about the same. Both clams and quahogs, *Venus Mercenaria*, use no oxygen from the water when tightly closed but the quahog takes up oxygen while slightly and invisibly open.

### THE METABOLIC RELATIONSHIP OF THE ACETONE BODIES.

By W. McK. MARRIOTT.

(From the Laboratory of Biological Chemistry, Washington University, St. Louis, Mo.)

Salts of acetoacetic,  $\beta$ -oxybutyric, and butyric acids were parenterally introduced into young pigs and into normal and phlorhizinized dogs with the following results:

Injected acetoacetic acid gave rise to an increase of both acetoacetic and  $\beta$ -oxybutyric acids in the circulating blood; the amount of  $\beta$ -oxybutyric acid being as great or greater than the diacetic acid present. Increased  $\beta$ -oxybutyric acid elimination in the urine followed. A comparison of results by the polarization and oxidation methods indicated that a small part of the  $\beta$ -oxybutyric acid excreted was the dextro rotatory form.

Injected  $\beta$ -oxybutyric acid gave rise to only a small increase in the acetoacetic acid content of the blood and little or no increase of the acetoacetic acid of the urine. Optically inactive  $\beta$ -oxybutyric acid was more readily burned than the levo rotatory form. The dextro component chiefly disappeared.

Injected butyric acid gave rise first to an increase of acetoacetic acid in the circulating blood and this was followed by an increase in  $\beta$ -oxybutyric acid.

From the experimental evidence, the conclusion may be drawn that the normal path of fatty acid catabolism is as follows:

Fatty acid  $\rightarrow$  acetoacetic acid  $\begin{cases} \nearrow d\text{-oxybutyric acid (readily burned).} \\ \searrow l\text{-oxybutyric acid (difficultly burned).} \end{cases}$

PHENOMENA OF NARCOSIS IN LEAVES OF THE WILD INDIGO  
(*Baptisia tinctoria*) AND THE CONSEQUENT PRODUCTION  
OF A NEW PHENOL, BAPTISOL.<sup>4</sup>

By ERNEST D. CLARK.

(From the Chemical Laboratory of the Cornell University Medical  
College, New York City.)

Preliminary experiments upon the leaves of the wild indigo showed that they were very sensitive to the action of certain volatile organic substances which caused a rapid change in color from green to jet black. The effects of different volatile substances upon the leaves were studied by subjecting them to narcosis under uniform experimental conditions. The time required for the leaves to turn completely black could thus be determined with some accuracy and it appeared to vary greatly with the different liquids used. The ordinary volatile antiseptics like chloroform, toluene, and ether were not nearly as active in causing the blackening as were substances of the ester type and acetone. Vapors of inorganic acids and ammonia produced non-typical brown colorations and rapid destruction of tissue. As reported by H. E. Armstrong and also by Mirande in another connection, no relation could be demonstrated between the structure or physical properties of the anaesthetics and their rate of action on the leaf. In general, the more chemically inert substances like esters were the most efficient in producing the typical effects. When the leaves were instantly killed by being plunged into boiling water they remained green for a long time and were not affected by any anaesthetic. Judging from this and other observations it seems probable that the blackening of these leaves is due to the activity of certain enzymes.

After considerable difficulty, the crystalline substance appearing on the blackened leaves in the form of a frost-like efflorescence was finally isolated in a pure state. Analysis showed the substance to be a new phenol of the formula  $C_{15}H_{12}O_6$ , melting at  $213^\circ$  (corrected). Freezing-point determinations and the preparation of the acetyl ( $C_{21}H_{18}O_8$ , m.p.  $189^\circ$ ) and benzoyl ( $C_{36}H_{24}O_8$ , m.p.  $183^\circ$ ) derivatives indicated that this formula is correct. The amount of pure substance was insufficient for the complete eluci-

<sup>4</sup> Read by title.

dation of its constitution but it was definitely ascertained that there are three hydroxyl groups and one methoxyl group. In the literature there are two other phenols of this composition, both isolated from plants, but they do not agree in any particular with this substance which we may call *baptisol*. The crystals of *baptisol* are small white needles which are nearly insoluble in water. They give a bright but evanescent cherry-red color with ferric chloride solution. With Millon's reagent *baptisol* produces a typical bright red coloration.

THE FORMATION OF GLUCOSE FROM CITRIC ACID IN  
DIABETES MELLITUS AND IN PHLORHIZIN  
GLYCOSURIA.

By ISIDOR GREENWALD.

(From the Harriman Research Laboratory, Roosevelt Hospital, New York.)

The administration of sodium citrate to phlorhizinized dogs and to a patient with diabetes mellitus was found to increase the excretion of glucose to an amount corresponding to a complete conversion of the six carbon atoms of the citric acid into glucose.

SUMMARY OF RESULTS OF ELECTROLYSIS OF PROTEIN AND  
THEIR DEGRADATION PRODUCTS.<sup>5</sup>

By JAMES P. ATKINSON.

(From the Chemical Laboratory, New York City Board of Health.)

I. After varied periods of electrolysis in the presence of 5 to 10 per cent sulphuric acid, solutions of whole protein cease to give the biuret reaction.

II. About 50 per cent of the total nitrogen from egg white,<sup>6</sup> Witte's peptone and hydrolyzed horse serum is obtained as ammonia within the time limits given.

III. Amino-acids (with the exception of glycocoll), uric acid

<sup>5</sup> Read by title.

<sup>6</sup> The electrolyses were run about forty-eight hours except in the case of uric acid which ran for sixty-seven hours and in the case of egg white which ran for 179 hours.

and urea after electrolysis yielded less than 50 per cent of their nitrogen as ammonia within the time limits of electrolysis given.

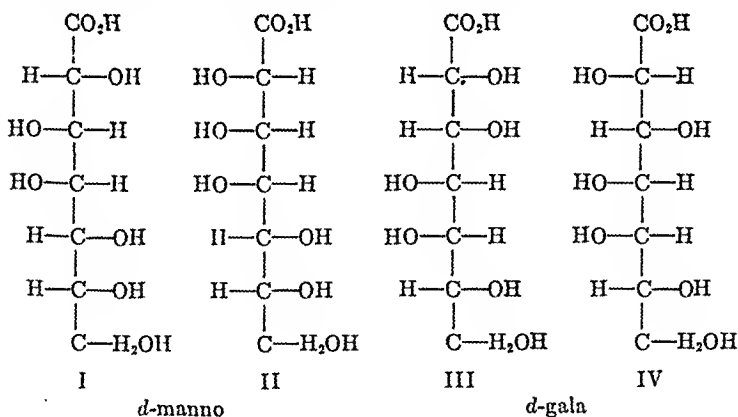
IV. The percentages of nitrogen obtained as ammonia from the amino-acids tested within the time limits of electrolysis given, are, roughly, inversely as their molecular weights.

## RESEARCHES ON THE HEPTOSES.

By GEORGE PEIRCE.

(From the Laboratory of Pharmacology, University of Wisconsin.)

*d*- $\alpha$ -Mannoheptite and *d*- $\alpha$ -galaheptite have a melting point of  $188^\circ$  and a specific rotation in saturated borax solution of  $+4.85^\circ$  and  $-4.35^\circ$  respectively. They form a compound melting at  $203^\circ$  uncorrected. Emil Fischer found that *d*- $\alpha$ -mannoheptite and *l*- $\alpha$ -mannoheptite formed a compound melting at  $203^\circ$  corrected. *d*- $\alpha$ -Mannoheptite is therefore evidently the antipode of *d*- $\alpha$ -galaheptite. The configurations of the heptioic acids formed from *d*-mannose and *d*-galactose are given below:



I and IV were made by Fischer, III by Kiliani and II can be made by heating I with pyridine. It was isolated as a phenylhydrazide. It will be seen that the acids represented by I and III give antipodes on oxidation or reduction of the end carbon atoms. I represents therefore the configuration of *d*- $\alpha$ -mannoheptioic acid and III that of *d*- $\alpha$ -galaheptioic acid. II and IV give the configuration of the  $\beta$ -acids.

This conclusion is strengthened by the fact that *d*- $\alpha$ -mannopentahydroxypimelic acid is apparently the antipode of *d*- $\alpha$ -galapentahydroxypimelic acid.<sup>7</sup>

## BIOLOGICAL OXIDIZABILITY AND CHEMICAL CONSTITUTION.

By HERBERT H. BUNZEL.

(Washington, D. C.)

The oxidase activities of the juices of shoots, foliage, and tubers of healthy and diseased (curly dwarf) potato plants were measured, using the following reagents: pyrocatechol, resorcin, hydrochinon, guaiacol, pyrogallol, phloroglucin,  $\alpha$ -naphthol, leucobase of malachite green, aloin, benzidine, tyrosine, phlorhizin, ortho-, meta-, and para-cresol, and ortho-, meta-, and para-toluidine.

The para compounds are more easily oxidized than the ortho and meta compounds; the meta compounds may be intermediate in their ease of oxidation or less oxidizable than the others. No matter what the derivation of the plant juice used is, the relative oxidizability of the various compounds is approximately the same.

## ALBUMINURIA FOLLOWING INGESTION OF PHENOLPHTHALEIN.

By J. L. HYDRICK.

(From the Laboratories of Bacteriology and Physiological Chemistry of Jefferson Medical College.)

In twenty different experiments in each of which, before beginning the experiment, the subject's urine showed no trace of albumin, by: (1) Robert's nitromagnesium test; (2) boiling and acetic acid test, and (3) Jolle's new ring test, a twenty-four-hour specimen of urine (a 24-hour specimen is important) collected after the administration of a 1 to 2 grain dose of phenolphthalein, in every one of these cases, gave, by the above mentioned three methods, positive tests for albumin. The amount of albumin varied from a trace up to 0.25 per cent by Esbach's quantitative method. The precipitate in many of the cases was tested and found to be insoluble.

<sup>7</sup> Abstract in *Science*, xxxviii, p. 677, 1913. Formulae III and IV in that abstract are obviously incorrect and should be replaced by formulae III and IV of the present abstract.

ble in alcohol. Phenolphthalein was demonstrable in the urine in traces. The albuminuria lasted from one to three days.

As to experiments on lower animals, two cats were given large doses of phenolphthalein for several days, their urine contained no demonstrable phenolphthalein nor albumin and their kidneys and other organs, after fixation, sectioning and examination, showed no pathologic changes.

Examinations of the feces, the condition of the alimentary canal, etc., following ingestion of phenolphthalein, all seem to show that the drug acts because its sodium salt is an irritant and produces purgation by: 1. Irritation of the intestinal mucous membrane by the unabsorbed portion and 2. Stimulation, by the absorbed portion of the intestinal nerve plexuses which regulate peristalsis.

#### A METHOD FOR THE DETERMINATION OF FAT IN SMALL AMOUNTS OF BLOOD.

By W. R. BLOOR.

*(From the Laboratory of Biological Chemistry, Washington University, St. Louis.)*

In this method a small amount of blood—ordinarily 2 cc.—is drawn from the vein with a graduated syringe and immediately emptied with stirring into 30–40 volumes of a mixture of 3 parts ethyl alcohol and 1 part ether. The protein is coagulated and the fat brought into solution by heating the mixture to the boiling point in a water bath, after which it is cooled, made up to 100 cc. and filtered. For the determination an aliquot portion containing 2–3 mgms. of fat is measured into a small beaker and saponified by evaporating to dryness with 2 cc. of normal sodium ethylate. Five cc. of the alcohol-ether mixture are added and after standing a few minutes the solution is poured with stirring into 100 cc. of distilled water, after which the beaker is rinsed out with the water. The solution is acidified with 10 cc. of 10 per cent HCl and compared with a standard fat solution by the use of the Richard's nephelometer.

The standard solution is an alcohol-ether solution of triolein containing 2–3 mgms. of fat in each 5 cc. For the comparison

5 cc. of this standard are measured into 100 cc. of water and the colloidal solution so obtained is flocced by the addition of 10 cc. of 10 per cent HCl.

Diagrams were presented showing increase in the fat content of blood after fat feeding (alimentary lipaemia), and the gradual rise of the fat content of the blood in starvation (up to five days), also of one experiment showing the rapid rise of blood fat during ether anaesthesia.

#### DETERMINATION OF CREATINE IN MUSCLE.

By LOUIS BAUMANN.

*(From the Chemical Research Laboratory, Medical Department, State University of Iowa, Iowa City.)*

A method for the determination of the sum of creatine and creatinine in muscle is proposed, which is simple, relatively rapid and accurate. The only reagent required other than the picric acid and the 10 per cent sodium hydrate used in Folin's colorimetric creatinine determination, is 5 N sulphuric acid.

#### A NEW RESPIRATORY CHAMBER.

By A. C. KOLLS AND A. S. LOEVENHART.

*(From the Pharmacological Laboratory of the University of Wisconsin.)*

The chamber described was designed for the purpose of determining the effect of reducing the oxygen of the respired air on the contents of the blood in erythrocytes and haemoglobin. The chamber is 3 feet wide, 3 feet long and  $1\frac{1}{2}$  feet high. It is made of No. 20 galvanized iron and contains three windows. It is constructed with a trap for getting the animals out without altering materially the atmosphere of the box.

We may report the results obtained with two young albino rabbits: (a) male, 1700 grams; (b) female, 1560 grams. These animals were placed in an atmosphere averaging 11.98 per cent of oxygen, 0.09 per cent carbon dioxide and 87.93 per cent nitrogen for 132 hours. Average relative humidity 35 per cent.

The blood counts before and after placing in the chamber were as follows:

RABBIT	HAEMOGLOBIN		ERYTHROCYTES	
	Before	After	Before	After
	per cent	per cent		
a	99.0	121.2	6,677,000	8,416,000
b	102.8	124.5	8,384,000	8,490,000

We conclude that a reduction of the oxygen tension entirely apart from barometric changes causes an increase in the erythrocytes and haemoglobin. That loss of water was not responsible for the blood changes is shown by the fact that the humidity in the box was above that of the outside air and also by the fact that rabbit (b) showed a marked increase in the haemoglobin without increasing his abnormally high count of erythrocytes. Furthermore, the blood contained a large proportion of basophilic macrocytes and presented further evidence that the changes in the blood count were due to stimulation of the bone marrow. We conclude that the bone marrow, like the respiratory center, responds to decrease in oxidation with stimulation and that the polycythemia and increase in haemoglobin noted at high altitudes is to be attributed to decreased oxygen tension in the bone marrow.

#### A RESPIRATION INCUBATOR FOR THE STUDY OF METABOLISM IN NEW-BORN AND PREMATURELY BORN INFANTS.

By JOHN R. MURLIN,

(From the Cornell University Medical College, New York City.)

A Frigas electric incubator of special dimensions was purchased<sup>\*</sup> and provided with an inner chamber (12 × 13 × 30 inches) made of heavy copper sheeting. The walls of the inner chamber are everywhere three inches distant from the inner asbestos wall of the incubator proper. This space can be both cooled and warmed. The former is accomplished by circulating cold water from an ice tank with a constant head of pressure through a coil of copper pipe; the latter by warm air from the electric heater circulated by a fan placed in the back of the incubator, which comes as a part of the instrument as purchased.

<sup>\*</sup> From Eimer and Amend, New York.

The inner chamber is closed by means of a glass head mounted in a brass frame. The frame is pressed upon a rubber gasket by means of screw clamps.

The respiration chamber is ventilated by means of the Benedict respiration machine. Oxygen is admitted from a small cylinder<sup>9</sup> containing, under 75 atmospheres' pressure, about 40 grams of oxygen. The oxygen is fed in automatically as described by Williams<sup>10</sup> for the small calorimeter.

The bed for the infant consists of a metal box ( $8 \times 4 \times 24$  inches) covered with rubber sheeting. A piece of kitchen linoleum serves as a receiving plate and the entire box as a receiving tambour. The slightest movements of the subject are transmitted as air waves through a wide rubber tube to a sensitive recording tambour on the top of the incubator. The kymograph is driven by a worm gear from the fan motor.

A stethoscope placed on the chest of the infant communicates every sound made by him to the ears of a nurse who also records the pulse and respirations every few minutes. The incubator is at present in use on the maternity service of J. Clifton Edgar in Bellevue Hospital.

### THE SPECIFIC RÔLE OF FOODS IN RELATION TO THE COMPOSITION OF THE URINE.

By N. R. BLATHERWICK.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University.)

The influence of individual foods upon the composition of the urine of healthy men was determined by placing them upon a uniform diet and then adding a single food to this basal ration, after normal values had been established. Results showed that some vegetables and fruits, because of their content of bases, are markedly efficient in reducing ( $\text{H}^+$ ) and ammonia excretion. They also produce conditions which enable the urines to dissolve uric acid. Potatoes, oranges, bananas, apples, raisins, cantaloupe and tomatoes caused such effects. The presence of certain or-

<sup>9</sup> Supplied by the Consolidated Dental Manufacturing Company, New York.

<sup>10</sup> Williams, H. B.: *Journ. Biol. Chem.*, xii, p. 321, 1912.

ganic acids in fruits leads to an increased acid production due to their incombustibility in the body. Cranberries, plums and prunes are examples. Benzoic acid, which is found in them, was also shown to produce like results. All urines of  $(\bar{H})^+$  less than 7.00 were capable of dissolving uric acid when opportunity offered. When the  $(\bar{H})^+$  was greater than  $1 \times 10^{-7}$  uric acid precipitated, *i.e.*, the urines were supersaturated. The average  $(\bar{H})^+$  of thirty vegetarian urines was found to be 6.63 as compared with the higher value of 6.03 obtained for normal urines by Henderson and Palmer.

### CREATINE- AND CREATININE-FREE FOODS.

By RITA K. CHESNUT.

(From the Lillian Massey Laboratory for Food Chemistry,  
University of Toronto.)

Analyses of some animal foods in order to determine the presence or absence of creatine and creatinine. The material was extracted with cold or warm water, the extract concentrated greatly on a water bath, filtered and creatinine tested for by Folin's colorimetric method; the creatine tested for by Benedict's method of heating the solution to dryness with hydrochloric acid and precipitating the protein with basic lead acetate.

Foods examined: Canned crabs, canned lobster, canned shrimp, canned clams, fresh oysters, canned scallops, canned caviar, calves' brains, sweetbreads, milk, bloaters' paste, anchovy paste, whitefish, halibut.

Creatine and creatinine absent in crabs, lobster, shrimps, clams, oysters, scallops, caviar, calves' brains, sweetbreads and milk. In bloaters' paste total creatinine is 0.12 per cent, in anchovy paste, 0.085 per cent, in whitefish, 0.19 per cent, and in halibut. 0.36 per cent.

Blank experiments were made comparing method of Benedict for determining creatine with the autoclave method of Benedict and Myers; results were concordant.

EFFECT OF GAS TAR ON OYSTERS.<sup>11</sup>

By PHILIP H. MITCHELL.

*(From the Biological Laboratory, Brown University.)*

Experiments show no noticeable effects of water-gas tar on oysters in constantly renewed sea-water. This is true in spite of the fact that large amounts of tar mixed with stagnant sea-water or small amounts injected into oysters which are kept in stagnant water do cause serious or fatal effects. Considerable quantities (1.5 cc.) may be put inside the shell of an oyster kept under conditions resembling those of its natural habitat without causing any effect. The harmlessness of the tar under these circumstances is due apparently to the ability of the oyster to rid itself of such foreign matter. In stagnant water the organism cannot be effectively washed out and effects, involving a loss of sensitiveness in the mantle, result. That consumption of the dissolved oxygen in the stagnant water by tar may have some effect on oysters is a possibility.

## THE EFFECT OF GLUCOSE ON AUTOLYSIS: A POSSIBLE EXPLANATION OF THE PROTEIN-SPARING ACTION OF CARBOHYDRATES.

By P. A. SHAFFER.

*(From the Laboratory of Biological Chemistry, Washington University, St. Louis, Mo.)*

Results of a series of antiseptic autolysis experiments with hashed dog-liver appear to indicate that the addition of 1 per cent of glucose to the mixtures under some conditions slightly inhibits the action of the proteolytic enzymes. It is believed that this may be a confirmation of an hypothesis held by the writer and already voiced in a somewhat different form by Ringer<sup>12</sup> that the "protein-sparing action of carbohydrates" is exerted through the influence of the concentration of glucose or of glycogen upon the enzymes of the cells.

The explanation of the "sparing action" on the supposition that sugar when present is merely burned by preference and thereby

<sup>11</sup> Read by title.<sup>12</sup> Ringer: *Journ. Biol. Chem.*, xii, p. 431, 1912.

makes unnecessary the catabolism of (body) protein, is wholly inadequate. Food-protein is not thus spared. The effect of carbohydrates in sparing body-protein in fever for instance<sup>13</sup> is probably in protecting the cell protein from digestion by the proteolytic enzymes of the cell, for when once attacked by the destructive enzymes, there is reason to believe that the autolytic products would behave like the similar products of protein absorbed from the intestine and their nitrogen be excreted promptly as urea. It seems to the writer that instead of supposing with Landerdren that body protein (in carbohydrate starvation) is catabolized to supply the essential sugar, it is more likely that so long as a normal concentration of sugar (or glycogen) is present in the cells the proteolytic enzymes are somewhat held in check and the strictly endogenous catabolism is restricted to its normal low value, but that when carbohydrate food is lacking and the sugar (or glycogen) concentration in the cells falls, an inhibition is removed, the proteolytic enzymes become correspondingly more active, a larger amount of cell protein is digested and the products like the products from food protein are in part converted into glucose.

It is believed that the autolysis experiments support this point of view.

#### THE PASSAGE OF ORGANIC SULPHATE FROM PLANT TO MEDIUM.<sup>14</sup>

By M. X. SULLIVAN.

(From the Bureau of Soils, U. S. Department of Agriculture.)

Wheat was germinated and placed on perforated aluminum discs, floated on water. With a change of water every two days, the seedlings were grown for ten days, with the seed in contact with the water for the first two days and subsequently with only the roots in contact with the water. In the combined solutions concentrated to a small volume, were found: creatinine by the creatinine-zinc-chloride method and color reactions; an aldehyde with a vanillin-like odor, and giving vanillin color reactions; a crystalline body containing sulphur and resembling cystine in crystalline shape and solubilities; and oily matter. The creat-

<sup>13</sup> Shaffer and Coleman: *Arch. of Int. Med.*, iv, p. 538, 1909.

<sup>14</sup> Read by title.

inine and the vanillin were found in the growing plants and must have passed from the plant to the medium. As yet cystine-like substance has not been found in the growing plant.

#### STUDIES ON CHICKEN FAT: VI. THE FACTORS INFLUENCING THE ACID VALUE OF THE CRUDE FAT.

By M. E. PENNINGTON, J. S. HEPBURN AND E. L. CONNOLLY.

*(From the Food Research Laboratory, Bureau of Chemistry, U. S. Department of Agriculture, Philadelphia.)*

The determination of the acid value of crude chicken fat has been used in this laboratory as a rapid and accurate measure of the decomposition of the fat. In the present research certain factors, which may influence the acidity of the crude fat, have been studied, and the following conclusions have been drawn: Low temperatures tend to retard the hydrolysis of the fat. The acidity is influenced to some extent by the quantity of fat, expressed as per cent of the body weight; other conditions being equal, a large quantity of fat is usually accompanied by a low acid value and vice versa. The acidity is also dependent on the degree of activity of the lipase. The degree of saturation of the fatty acids, measured by the iodine number, and the titre of the insoluble fatty acids, apparently, are without influence on the acidity. The study reported would tend to confirm the reliability of the acidity of crude chicken fat as an index of decomposition.

The influence exerted by the various factors, just enumerated, while of interest from the viewpoint of acquiring knowledge of biochemical processes and especially of those relating to the decomposition of fat do not vitiate the practical usage of this method as it has been applied by this laboratory.

#### THE INFLUENCE OF RESTRICTED RATIONS ON GROWTH.<sup>15</sup>

By E. B. HART AND E. V. McCOLLUM.

*(From the Laboratory of Agricultural Chemistry of the University of Wisconsin.)*

In nutrition experiments with herbivora it has been shown that rations restricted to the wheat plant will not allow a complete

<sup>15</sup> Read by title.

cycle of life, although growth can be secured. With rats<sup>16</sup> growth is suspended after a period of about one hundred days, where the ration is limited to the wheat kernel, its proteins and a salt mixture similar to that of milk. On the wheat kernel and its proteins alone little growth can be induced.

Further work with swine confirmed these results in every particular. Little or no growth could be obtained where the animals received a ration of wheat and its proteins and when they were limited to this ration and distilled water. The same results were secured with a ration of corn-meal and gluten feed.

When, however, a salt mixture consisting of potassium phosphate, potassium citrate and calcium lactate was added to the wheat ration, a period of considerable growth was obtained, but ceased when the animals reached a weight of approximately 100 pounds. At this point maintenance prevailed for a period of three months after which the animals began to lose weight.

On rations of corn-meal, gluten feed and a salt mixture of potassium phosphate and calcium lactate growth with swine was constant and continued, producing animals of 200 to 300 pounds' weight in six months. This is somewhat below the normal curve of growth on mixed rations and a range for exercise, but there were periods of growth where the rate was a normal one.

These results are not in harmony with the theory that the failure of swine to grow on corn alone is due entirely to the "incomplete" nature of its protein content.

Further, the contrast between the results secured with wheat and corn are tentatively explained on the assumption that there exists in the wheat kernel some toxic substance. Experiments in feeding germinated wheat to young rats indicate that the injurious character of the wheat kernel as the sole nutrient for this species is at least markedly diminished during the germinating process. Further experiments in this direction are in progress.

<sup>16</sup> E. V. McCollum and Marguerite Davis: *Proc. Biol. Chemists*, Cleveland Meeting, January, 1913.

PRODUCTION OF AMMONIA BY HERBIVORA AS A PROTECTION  
AGAINST ACIDOSIS.<sup>17</sup>

BY E. B. HART AND V. E. NELSON.

*(From the Laboratory of Agricultural Chemistry of the University of Wisconsin.)*

The opinion is quite common among physiological chemists that omnivora and carnivora can effectively protect themselves against acidosis in the tissues by ammonia production, but that herbivora have no such power, or at least only to a limited extent. This latter view rests primarily upon records secured with rabbits. In our nutrition experiments with calves and cows evidence was secured that this species, although normally living upon an alkaline diet, such as milk, or grain and roughage, will raise the ammonia output in the urine as the acid character of the ration is increased.

A calf fed normal milk produced ammonia nitrogen in the urine equivalent to 5 to 8 per cent of the total urinary nitrogen output. When the milk was made acid with hydrochloric acid in an amount equivalent to 21 cc. of N HCl per pound of milk and which gave a decided preponderance of acids over bases in the ration, the ammonia-nitrogen output was raised to 22 per cent of the total urinary nitrogen.

Further, this class of animals was capable of such control on comparatively low protein rations. In an experiment where mature cows were maintained on a relatively low plane of protein intake—nutritive ration 1:8—but on a ration made acid with mineral acids, ammonia nitrogen in the urine was as high as 24 per cent of the total urinary nitrogen output. With the same ration, but minus the mineral acids the ammonia nitrogen produced in the urine was reduced to 2 to 3 per cent of the total urinary nitrogen.

<sup>17</sup> Read by title.

THE INFLUENCE OF RESTRICTED RATIONS ON  
REPRODUCTION.<sup>18</sup>

BY E. B. HART, E. V. McCOLLUM AND H. STEENBOCK.

*(From the Laboratory of Agricultural Chemistry of the University  
of Wisconsin.)*

In our previously reported work<sup>19</sup> with "balanced" but restricted rations fed herbivora it was shown that the entire wheat plant failed to allow the reproduction of normal, vigorous offspring. With rations balanced from the corn plant a perfect cycle could be obtained. In continued studies with cows, rations balanced from the corn grain and wheat straw produced weak under-sized offspring. When, however, a salt mixture made of the lactates and citrates of potassium, magnesium and calcium was added to the ration and made equivalent in base intake to that of a ration completely compounded from the corn plant, a complete and normal cycle was produced. Substitution of carbonates for the salts of organic acids was not successful in correcting the defects of the ration. Apparently on the ration of corn grain and wheat straw either a lack of an adequate supply or more probably a proper physiological balance of mineral substances was the primary cause for the production of weak or dead progeny.

On rations balanced from the wheat grain and wheat straw—rations that are always disastrous for the offspring—but to which the same organic salt mixture mentioned above was added some increase in the length of intra-uterine life was obtained, but the offspring were weak and under-sized, living but a short time. The mere substitution of the wheat grain for the corn grain introduced a disturbing factor. We are inclined to believe at present that this factor is of the nature of toxicity.

CALCIUM METABOLISM AFTER THYRO-PARATHYROIDECTOMY.<sup>20</sup>

BY F. T. STEWART, OLAF BERGEIM, AND P. B. HAWK.

*(From the Laboratory of Physiological Chemistry of Jefferson Medical  
College.)*

The metabolism of calcium was studied in a patient (man) after complete removal of thyroid and parathyroid glands. A slight

<sup>18</sup> Read by title.<sup>19</sup> Research Bulletin No. 17, Wisconsin Exp. Station.<sup>20</sup> Read by title.

retention of calcium, 0.3854 gram  $\text{CaO}$  in the ten-day period, was noted. The urinary  $\text{Ca}$  excretion was low, averaging 0.0134 gram per day on a daily ingestion averaging 1.6736 grams of  $\text{CaO}$ . The calcium content of the blood was slightly lower than the calcium value for normal blood, and slight increase was observed during the period of study.

No symptoms of tetany were noted in the patient who survived operation thirty-nine days.

VARIATIONS IN THE HYDROGEN ION CONCENTRATION OF  
THE URINE OF MAN ACCOMPANYING FASTING AND  
THE LOW- AND HIGH- PROTEIN REGENERATION  
PERIODS.<sup>21</sup>

BY PAUL E. HOWE AND P. B. HAWK.

*(From the Laboratory of Physiological Chemistry, University of Illinois.)*

A study was made of the variation in the concentration of hydrogen ion in the urine of a man throughout a seven-day fast, the subsequent four-day low protein feeding period and in another period of five days in which the individual received a high protein diet similar to that ingested in the normal period previous to the fast. The concentration of hydrogen ion rose during the first two days of the fast from the normal value of  $P_H$  7.34 to 5.63 on the third day and then remained at practically this value through the last four days. As the result of feeding the concentration of hydrogen ion fell gradually in the four days of low protein feeding (one-fourth the original diet) from  $P_H$  5.84 on the last day of the fast to 7.87 on the fourth day of this period. Upon the resumption of the high protein diet the hydrogen ion concentration continued to fall for three days, reaching the low value of  $P_H$  9.00, and then returned to the normal concentration during the last two days of the experiment. There was no direct relation between the hydrogen ion concentration and the ammonia output.

<sup>21</sup> Read by title.

SOURCES OF SURFACE TENSION IN STRIATED MUSCLE.<sup>22</sup>

By WILLIAM N. BERG.

(Washington, D. C.)

Throughout the works of Bernstein, Jensen, and Macallum on muscle contraction, the assumption is made or implied, that the chemical changes taking place in an active muscle are such as to give rise to a surface tension between the contractile units and the adjacent lymph (sarcoplasm), as if a difference in chemical composition or in concentration between two adjacent regions necessarily causes surface tension at the surface of contact.

Methods, developed by others, for the calculation of the surface tension between two liquids or solutions are applied to solutions of biological substances. Reasons are advanced showing that the sources of surface tension in muscle are not nearly as numerous as might be supposed. The range between the maximum and minimum surface tension in muscle is evidently very narrow. The maximum value already indicated, *i.e.*, about 85 dynes per centimeter for the surface tension at the beginning of the contraction phase, is shown to be almost impossibly high. The actual value is at present unknown from direct measurement on muscle. It is probably a small fraction of 85 dynes, if surface tension plays any rôle at all in muscle contraction.

In certain aqueous solutions changes in concentration do not change the surface tension and in certain cases two solutions in contact with one another may differ very greatly in concentration of a common solute with almost zero surface tension between them.

THE MAXIMUM SURFACE TENSION IN STRIATED MUSCLE.<sup>23</sup>

By WILLIAM N. BERG.

(Washington, D. C.)

At the moment when a striated muscle begins to contract against an external resistance, the surface tension between the contractile

<sup>22</sup> Transferred with the author's consent to the program of the Physiological Society.

<sup>23</sup> Transferred with the author's consent to the program of the Physiological Society.

units and their surrounding medium cannot be higher than about 85 dynes per centimeter.

Jensen's and Macallum's statements to the effect that the surface tension on certain forms of living matter may be as high as 6000 dynes per centimeter are, in all probability, erroneous.

The author points out an error in his own calculations already published and reaffirms the belief in the substantial correctness of the main conclusion: *i.e.*, that the lifting power of striated muscle cannot be explained on the basis that a working muscle is a mechanism that can transform a quantity of surface energy equivalent to the external work done.

#### TRANSFUSION OF BLOOD IN SEVERE DIABETES MELLITUS.<sup>24</sup>

By R. T. WOODYATT AND B. O. RAULSTON.

(From the Otho S. A. Sprague Memorial Institute Laboratory of Clinical Research, Rush Medical College, Chicago.)

Five hundred cc. of blood from an arm vein of a healthy man was transfused into a corresponding vein of a brother two years younger suffering from a diabetes of severe type. The transfusion was followed by a pronounced increase of the sugar, ammonium, acetone bodies and nitrogen of the urine, the diabetic quotient (Q) rising from 50 to 61.

$$Q = \frac{\text{urinary glucose} \times 100}{\text{ingested carbohydrate} + (\text{urinary N} \times 3.65)}$$

#### PHLORHIZIN GLYCOSURIA BEFORE AND AFTER THYROIDECTOMY.

By GRAHAM LUSK.

#### STUDIES IN DIABETES: (1) THE EFFECT OF DIFFERENT COMPOUNDS ON GLYCOGENESIS; (2) THE MECHANISM OF ANTIKETOGENESIS.

By A. I. RINGER AND E. M. FRANKEL.

#### CHANGES IN FATS DURING ABSORPTION<sup>25</sup>

By W. R. BLOOR.

<sup>24</sup> Transferred with the author's consent to the program of the Physiological Society.

<sup>25</sup> Published in full in the *Journal of Biological Chemistry*, xvi, p. 517, 1914.

ANAPHYLAXIS IN THE CAT AND OPOSSUM.

By C. W. EDMUNDS.

VIVIDIFFUSION; REPORT ON PRELIMINARY RESULTS.

By J. J. ABEL, L. S. ROWNTREE, AND B. B. TURNER.

A METHOD OF DIALYZING NORMAL CIRCULATING BLOOD  
AND SOME OF ITS APPLICATIONS.

By C. L. V. HESS AND H. MCGUIGAN.

A BIOLOGICAL TEST FOR IODINE IN THE BLOOD.

By A. WOELFEL AND A. L. TATUM.

FURTHER STUDIES OF THE EXCRETION OF ACIDS.

By L. G. HENDERSON AND W. W. PALMER.

METABOLISM IN DIABETES INSIPIDUS.<sup>26</sup>

By S. BOOKMAN.

ABSORPTION OF ANTITOXIN FROM SOLUTIONS CONTAINING  
DIFFERENT PERCENTAGES OF PROTEIN.<sup>27</sup>

By W. H. PARK, E. J. BANZHAF, AND L. W. FAMULENER.

THE CARBOHYDRATE TOLERANCE OF FEEBLE-MINDED  
CHILDREN ESPECIALLY OF THE MONGOLIAN TYPE.

By A. W. PETERS AND M. E. TURNBULL.

PROTEIN METABOLISM IN INDIVIDUALS WITH EXFOLIATIVE  
CONDITIONS OF THE SKIN.

By A. I. RINGER AND G. W. RAIZISS.

A HITHERTO UNKNOWN CONSTITUENT OF NERVE CELLS.

By A. B. MACALLUM AND J. P. COLLIP.

A NOTE ON THE CHEMICAL CONSTITUENTS OF THE CERE-  
BRO-SPINAL FLUID IN CERTAIN CASES OF INSANITY.<sup>28</sup>

By H. M. ADLER AND B. H. RAGLE.

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<sup>26</sup> Read by title.

<sup>27</sup> Transferred with the author's consent from the program of the Pharmacological Society.

<sup>28</sup> Read by title.

ON THE ESTIMATION OF MINUTE QUANTITIES OF  
PHOSPHORUS.

By A. E. TAYLOR AND C. W. MILLER.

THE NERVE CONTROL OF THE THYROID GLAND.<sup>29</sup>

By C. G. FAWCETT AND J. A. RAHE.

THE DETERMINATION OF BLOOD SUGAR  
(DEMONSTRATION).

By P. A. SHAFFER.

A SIMPLIFIED AND INEXPENSIVE OXIDASE APPARATUS  
(DEMONSTRATION).

By H. H. BUNZEL.

EXPERIMENTAL HYDROCHLORIC ACID INTOXICATION.<sup>30</sup>

By S. BOOKMAN.

FURTHER STUDIES OF THE QUANTITATIVE CHEMICAL COM-  
POSITION OF URINARY CALCULI.<sup>30</sup>

By J. ROSENBLOOM.

ON THE QUANTITATIVE CHEMICAL COMPOSITION OF GALL  
STONES.<sup>30</sup>

By J. ROSENBLOOM.

METABOLISM STUDIES IN A CASE OF FAMILY PERIODIC  
PARALYSIS.<sup>30</sup>

By J. ROSENBLOOM.

THE PRODUCTION OF GLYCOSURIA BY ZINC SALTS.<sup>31</sup>

By W. SALANT AND M. KAHN.

FURTHER OBSERVATIONS OF CAFFEINE GLYCOSURIA.<sup>31</sup>

By W. SALANT AND M. KAHN.

STUDIES UPON THE LONG CONTINUED FEEDING OF  
SAPONINE.<sup>31</sup>

By C. L. ALSBERG AND C. S. SMITH.

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<sup>29</sup> Presented by S. P. Beebe.<sup>30</sup> Read by title.<sup>31</sup> Transferred with the author's consent from the program of the Pharmacological Society.

## RESEARCHES ON PURINES. XIV.<sup>1</sup>

### ON 2,8-DIOXY-1,7,9-TRIMETHYLPURINE, AN ISOMER OF CAFFEINE, AND 2,8-DIOXY-1,7-DIMETHYLPURINE, AN ISOMER OF THEOBROMINE.

By CARL O. JOHNS.

(From the Sheffield Laboratory of Yale University.)

(Received for publication, December 2, 1913.)

Theoretically only two 2,8-dioxy-trimethylpurines can exist in which all of the methyl groups are attached to nitrogen atoms. One of these has already been described, namely, 2,8-dioxy-3,7,9-trimethylpurine (I).<sup>2</sup> Emil Fischer prepared this compound by methylating 2,8-dioxy-3,7-dimethylpurine (II).<sup>3</sup>

The writer has found that when 2,8-dioxy-1,9-dimethylpurine (XIV)<sup>4</sup> was alkylated with dimethyl sulphate a good yield of 2,8-dioxy-1,7,9-trimethylpurine (XI) was obtained. This compound is of interest because it is isomeric with caffeine (III). It melts at 240°C. which is only 1° above the melting point of caffeine. It differs from caffeine in that it is much more soluble in water and does not give a purple color when it is evaporated with chlorine water and the residue is moistened with ammonia.

As is well known, caffeine produces diuresis and is a powerful heart stimulant. As soon as opportunity offers the physiological and pharmacological properties of 2,8-dioxy-1,7,9-trimethylpurine will be investigated.

When 2,8-dioxy-1-methylpurine (IX)<sup>5</sup> was alkylated with dimethyl sulphate a 2,8-dioxy-dimethylpurine was obtained. This could be either 2,8-dioxy-1,7-dimethylpurine (VIII) or 2,8-dioxy-1,9-

<sup>1</sup> Johns and Baumann: this *Journal*, xvi, p. 135, 1913. The present investigation was aided by a grant from the Bache fund.

<sup>2</sup> Emil Fischer: *Ber. d. deutsch. chem. Gesellsch.*, xxx, p. 1853, 1897.

<sup>3</sup> Emil Fischer: *ibid.*, xxviii, p. 2487, 1895; xxx, p. 1851, 1897.

<sup>4</sup> Johns: this *Journal*, xiv, p. 5, 1913.

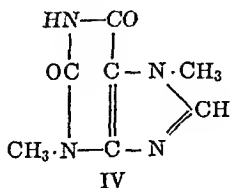
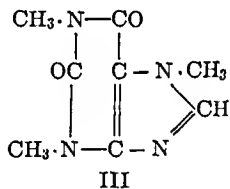
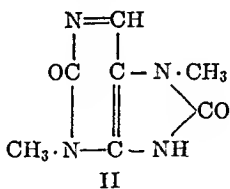
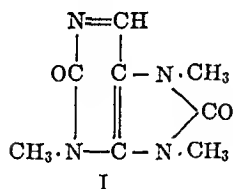
<sup>5</sup> Johns: *ibid.*, xi, p. 399, 1912.

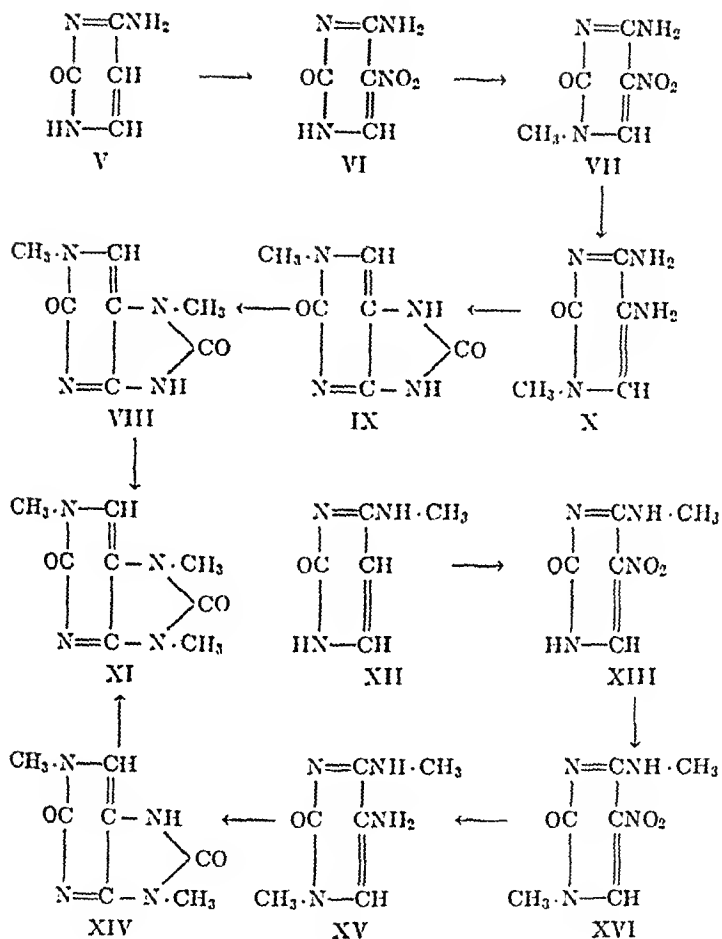
dimethyl-purine (XIV). The latter compound crystallizes in nacreous plates that do not decompose at 350°C. The compound which we obtained by alkylating 2,8-dioxy-1-methylpurine crystallized in acicular prisms that charred below 320°C. and which were converted to 2,8-dioxy-1,7,9-trimethylpurine (XI) by further methylation. Hence, it was considered to be 2,8-dioxy-1,7-dimethylpurine (VIII) which is a new isomer of theobromine (IV). In some experiments we also obtained small quantities of 2,8-dioxy-1,9-dimethylpurine (XIV) which was identified by its characteristic nacreous plates.

During the course of these researches it was found that instead of using methyl iodide for alkylating purines dimethyl sulphate could be employed. By making this change the yields of the methylated purines were usually increased, much time was saved and the use of sealed tubes was obviated.

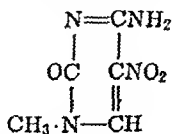
The various steps leading from cytosine (V) and 6-methyleytosine (XII) to 2,8-dioxy-1,7,9-trimethylpurine (XI) are shown by means of the accompanying structural formulae.

These researches will be continued.





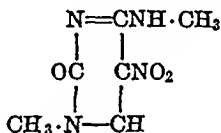
## EXPERIMENTAL PART.

*2-Oxy-3-methyl-5-nitro-6-aminopyrimidine.*<sup>6</sup><sup>6</sup> Johns: this *Journal*, xi, p. 75, 1912.

This pyrimidine was first prepared by heating the potassium salt of nitrocytosine with methyl iodide in a sealed tube at 100°C. It can be prepared more conveniently by using dimethyl sulphate as the alkylating agent.

Five grams of nitrocytosine were dissolved in 50 cc. of warm water containing 1.8 grams of potassium hydroxide and the solution was cooled to room temperature. Five grams of dimethyl sulphate were then added and the mixture was shaken until it showed an acid reaction with litmus. The 2-oxy-3-methyl-5-nitro-6-aminopyrimidine separated in a crystalline form. The yield was 4 grams.

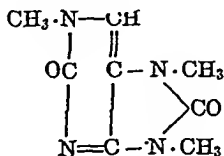
*2-Oxy-3-methyl-5-nitro-6-methylaminopyrimidine.*<sup>7</sup>



This compound was also prepared by using dimethyl sulphate as the alkylating agent instead of methyl iodide.

When 5 grams of 2-oxy-5-nitro-6-methylaminopyrimidine dissolved in 30 cc. of a normal sodium hydroxide solution were shaken with 3.2 grams of dimethyl sulphate 4.7 grams of 2-oxy-3-methyl-5-nitro-6-methylaminopyrimidine were obtained.

*2,8-Dioxy-1,7,9-trimethylpurine.*

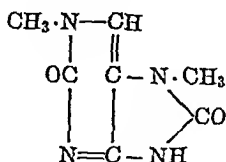


Five grams of 2,8-dioxy-1,9-dimethylpurine<sup>8</sup> were dissolved in 30 cc. of a normal sodium hydroxide solution. Four grams of dimethyl sulphate were added and the mixture was shaken thoroughly until it was acid to litmus. The resulting solution was

<sup>7</sup> Johns: this *Journal*, xiv, p. 3, 1913.

<sup>8</sup> Johns: *ibid.*, xiv, p. 5, 1913.

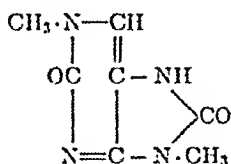


*2,8-Dioxy-1,7-dimethylpurine.*

Two and seven-tenths grams of 2,8-dioxy-1-methylpurine<sup>9</sup> were dissolved in 22 cc. of warm water containing 0.65 gram of sodium hydroxide and the solution was cooled to room temperature whereupon 2.2 grams of dimethyl sulphate were added. This mixture was shaken thoroughly and in less than ten minutes a crystalline substance began to form. When the mixture became acid to litmus the precipitate was filtered off. The filtrate was evaporated to almost dryness, then made alkaline with ammonia and finally evaporated to dryness. The residue, after washing with a little water and alcohol, was added to the crystals already obtained. The total yield was about 60 per cent of the weight calculated for a dioxy-dimethyl-purine. This substance was recrystallized from water. It gave an asbestos-like mat that was composed of acicular crystals. These did not melt but began to char below 320°C. They dissolved readily in hot water and crystallized again on cooling the solution. They were slightly soluble in hot alcohol and insoluble in boiling benzene. They differed from the crystals of 2,8-dioxy-1,9-dimethylpurine, which are nacreous plates and which do not decompose at 350°C. Moreover, during repeated crystallizations the needles did not show any tendency to form plates nor did the nacreous plates of 2,8-dioxy-1,9-dimethylpurine form needles. On further alkylation with dimethyl sulphate the needles formed 2,8-dioxy-1,7,9-trimethylpurine. Hence, the needles must be the isomer of 2,8-dioxy-1,9-dimethylpurine, or 2,8-dioxy-1,7-dimethylpurine.

	Calculated for C <sub>7</sub> H <sub>5</sub> O <sub>2</sub> N <sub>3</sub>	I	Found: II
N.....	31.11	31.31	31.06

<sup>9</sup> Johns: this *Journal*, xi, p. 398, 1912.

*The formation of 2,8-dioxy-1,9-dimethylpurine.<sup>10</sup>*

In one of the experiments in which the methylation of 2,8-dioxy-1-methylpurine was carried out in the manner described above a mixture of the naereous plates, which are characteristic of 2,8-dioxy-1,9-dimethylpurine, and needles of 2,8-dioxy-1,7-dimethylpurine was obtained. These compounds were separated by fractional crystallization from a water solution, the plates crystallizing out before the needles. Hence, although the methylation of 2,8-dioxy-1-methylpurine, as previously described, produces chiefly 2,8-dioxy-1,7-dimethylpurine, small quantities of 2,8-dioxy-1,9-dimethylpurine may also be formed.

*2,8-Dioxy-1,7,9-trimethylpurine from 2,8-dioxy-1,7-dimethylpurine.*

Eight-tenths gram of 2,8-dioxy-1,7-dimethylpurine was dissolved in 4.8 cc. of normal sodium hydroxide solution and 1 gram of dimethyl sulphate was added. The solution was shaken until it became acid to litmus and then evaporated to dryness. The residue was treated with excess of ammonia and then dried thoroughly at 100°C. The purine was then extracted with chloroform, the latter solvent was evaporated and the residue was recrystallized from alcohol. The substance, thus obtained, melted at 240°C. and agreed in all respects with 2,8-dioxy-1,7,9-trimethylpurine and when mixed with a pure sample of the latter compound the mixture also melted at 240°C.

	Calculated for $\text{C}_8\text{H}_{10}\text{O}_2\text{N}_4$	Found:
N.....	28.86	28.79

<sup>10</sup> Johns: this *Journal*, xiv, p. 5, 1913.



## PROTOZOAN PROTOPLASM AS AN INDICATOR OF PATHOLOGICAL CHANGES. III. IN FATIGUE.

By FRANK P. UNDERHILL AND LORANDE LOSS WOODRUFF.

(From the Sheffield Laboratory of Physiological Chemistry and the Osborn Zoological Laboratory, Yale University, New Haven, Connecticut.)

(Received for publication, December 3, 1913.)

Numerous investigations have demonstrated that the products formed during muscular activity exert a specific influence upon the function of certain of the higher forms of animal life. The work of Lee<sup>1</sup> in particular has pointed out that among these substances may be found carbon dioxide, monopotassium phosphate and paralaetic acid. According to this author fatigue substances in small quantity have a physiological action which is exactly the reverse of that of the same substance in larger quantity. In the earlier stages of muscular work the fatigue substances are present in small quantity, in the later stages in large quantity. Correspondingly in the early stages there is augmentation, in the later stages there is depression or fatigue.

The modifications occurring during fatigue are presumably so slightly different from the normal that it is *a priori* improbable that extracts of fatigued muscles would contain substances capable of exerting a marked influence on *Paramecium* protoplasm. In view of this it is obvious that to subject paramecia to the influence of fatigued muscle extracts is to place upon this organism one of the most delicate tests possible as an indicator of chemical change.<sup>2</sup>

In our experiments with fatigue products the source of these substances was the muscles of the frog. The hind legs of a very large frog, kept with skin intact, were entirely separated from each other, placed in a clamp, and one stimulated through the

<sup>1</sup> Lee: *Harvey Lectures*, 1906, where the literature is cited.

<sup>2</sup> Woodruff and Underhill: Protozoan Protoplasm as an Indicator of Pathological Changes. I. In Nephritis, this *Journal*, xv, p. 385, 1913; Underhill and Woodruff: Protozoan Protoplasm as an Indicator of Pathological Changes. II. In Carcinoma, *ibid.*, xv, p. 401, 1913.

sciatic nerve with increasing strengths of an interrupted induction current for an hour at a rate of about fifteen times per minute. This length of stimulation sufficed to put the muscles in a condition where they would no longer respond to the strongest stimulus through the nerve. The second leg was not stimulated. Both the stimulated and the unstimulated muscles were kept moist with Ringer's solution. In the trials with frogs I and II (see table) extracts were made of the gastrocnemius muscle only, but in the eight other frogs this muscle together with all the muscles of the thigh were employed.

The extracts were prepared in accordance with the methods outlined in our first paper of these studies.<sup>3</sup> The solutions were faintly acid to litmus. The tests of the extracts on the rate of division of a pedigreed race of *Paramecium* were also made according to the methods previously described. Trials were made with various strengths of solution. In undiluted solutions the paramecia would survive for a day or two only, extracts from both the stimulated and unstimulated muscles behaving alike in this respect. The optimum strength of extract was obtained by diluting one part of the original extract with three volumes of water. The results obtained with solutions weaker than the dilution 1:3 in every case corroborated those obtained with this dilution and accordingly the data obtained from 1:3 solutions only are presented in the table.

Comparison of the figures for the extracts of the stimulated and unstimulated legs demonstrates that in the great majority of cases the extract of the fatigued muscles produces no change in the division rate of *Paramecium*.

#### CONCLUSIONS.

Extracts prepared from fatigued muscles produce no change in the division rate of *Paramecium* as compared with that obtained with extracts of normal muscles.

Inasmuch as it has been demonstrated that paramecia are capable of detecting chemical changes, for example, in nephritis and in carcinoma, it is justifiable to conclude that the character of the chemical changes occurring in fatigued muscle must differ only slightly from the normal.

<sup>3</sup> Woodruff and Underhill: *loc. cit.*

TABLE I.

Comparison of the division rate of *Paramecium* in extracts of normal and of fatigued muscles.<sup>4</sup>

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
Frog I Normal	A.....	8	13	14	19	29
	B.....	8	13	21	29	36
	Total.....	16	26	35	48	65
Frog I Fatigued	A.....	8	18	25	33	39
	B.....	9	16	23	34	39
	Total.....	17	34	48	67	78
Frog II Normal	A.....	8	13	19	31	37
	B.....	7	14	22	32	39
	Total.....	15	27	41	63	76
Frog II Fatigued	A.....	7	15	22	32	39
	B.....	6	13	22	31	35
	Total.....	13	28	44	63	74
Frog III Normal	A.....	4	10	16	22	29
	B.....	4	12	20	26	32
	Total.....	8	22	36	48	61
Frog III Fatigued	A.....	6	8	19	24	35
	B.....	6	7	15	23	31
	Total.....	12	15	34	47	66
Frog IV Normal	A.....	4	9	20	24	29
	B.....	3	5	10	17	24
	Total.....	7	14	30	41	53
Frog IV Fatigued	A.....	3	11	15	19	21
	B.....	4	13	19	23	27
	Total.....	7	24	34	42	48
Frog V Normal	A.....	2	8	12	12	15
	B.....	6	9	11	13	14
	Total.....	8	17	23	25	29
Frog V Fatigued	A.....	4	10	14	18	22
	B.....	5	12	15	18	20
	Total.....	9	22	29	36	42

<sup>4</sup> The experiments on the various frogs were conducted at different times and accordingly under slightly different conditions of temperature, etc. Therefore the absolute number of divisions obtained in the extracts from different frogs are not comparable.

## Fatigue

TABLE I—Continued.

SUBSTANCE TESTED	CULTURE	NO. OF DIVISIONS AFTER DAYS				
		1	2	3	4	5
Frog VI Normal	A.....	8	20	29	38	42
	B.....	9	18	28	37	43
	Total.....	<u>17</u>	<u>38</u>	<u>57</u>	<u>75</u>	<u>85</u>
Frog VI Fatigued	A.....	7	18	26	32	39
	B.....	8	19	30	36	44
	Total.....	<u>15</u>	<u>37</u>	<u>56</u>	<u>68</u>	<u>83</u>
Frog VII Normal	A.....	8	17	28	38	45
	B.....	8	18	29	37	45
	Total.....	<u>16</u>	<u>35</u>	<u>57</u>	<u>75</u>	<u>90</u>
Frog VII Fatigued	A.....	8	17	25	33	41
	B.....	9	17	27	36	43
	Total.....	<u>17</u>	<u>34</u>	<u>52</u>	<u>69</u>	<u>84</u>
Frog VIII Normal	A.....	8	19	28	36	45
	B.....	6	17	28	35	43
	Total.....	<u>14</u>	<u>36</u>	<u>56</u>	<u>71</u>	<u>88</u>
Frog VIII Fatigued	A.....	8	20	29	37	44
	B.....	7	16	26	33	40
	Total.....	<u>15</u>	<u>36</u>	<u>55</u>	<u>70</u>	<u>84</u>
Frog IX Normal	A.....	8	16	27	37	45
	B.....	8	18	29	38	48
	Total.....	<u>16</u>	<u>34</u>	<u>56</u>	<u>75</u>	<u>93</u>
Frog IX Fatigued	A.....	8	16	25	32	40
	B.....	8	17	29	34	41
	Total.....	<u>16</u>	<u>33</u>	<u>54</u>	<u>66</u>	<u>81</u>
Frog X Normal	A.....	8	17	27	36	43
	B.....	9	19	27	35	42
	Total.....	<u>17</u>	<u>36</u>	<u>54</u>	<u>71</u>	<u>85</u>
Frog X Fatigued	A.....	8	16	27	33	44
	B.....	8	17	28	36	44
	Total.....	<u>16</u>	<u>33</u>	<u>55</u>	<u>69</u>	<u>88</u>

# THE CHOLESTEROL CONTENT OF CANCERS IN RATS.

By C. B. BENNETT.

(From the Rudolph Spreckels Physiological Laboratory of the University of California.)

(Received for publication, December 10, 1913.)

This work was undertaken at the suggestion of Dr. Burnett, to find out if the injection of cholesterol into rats made any permanent difference in the amount of cholesterol in the cancers of these rats. The strain of cancer used was the Flexner-Jobling rat carcinoma, originally obtained from Dr. Peyton Rous of the Rockefeller Institute, New York. The cholesterol was not injected into the cancers in these experiments, but was placed subcutaneously in the other side of the animals. The rats taken were those experimented upon by Dr. Burnett which he very kindly allowed me to use. Each rat received, repeatedly, 1 cc. of a 2 per cent solution of cholesterol in sodium oleate. Nine or ten injections were made, during a period of about three weeks.<sup>1</sup> It was soon found that a direct comparison between the injected and uninjected rats was impossible owing to the fact that there was considerable individual variation among those treated exactly alike. The following deductions, however, seem to be indicated by the findings:

1. The injection of cholesterol into rats does not very markedly affect the cholesterol content per gram of cancer tissue.
2. The cholesterol content of cancers increases with the age of the cancers, a fact of some interest when it is remembered that Wacker found the same thing true for normal tissues.<sup>2</sup>
3. The outside, or actively growing part of the cancer contains less cholesterol than the central portion.
4. If two cancers are of the same age, that one which is very markedly the smaller is apt to contain more cholesterol per gram

<sup>1</sup> For further details of treatment, see Theodore C. Burnett: *Proc. of the Soc. for Exp. Biol. and Med.*, xi, p. 42, 1913.

<sup>2</sup> Leonhard Wacker: *Zeitschr. f. physiol. Chem.*, lxxx, p. 383, 1912.

of tissue, probably because it contains much less actively growing tissue.

## EXPERIMENTAL.

RAT	INOCU- LATED	KILLED	REMARKS	WEIGHT OF TUMOR	CHOLE- STEROL CON- TENT PER GRAM OF TISSUE
				grams	mgm.
1	Aug. 13	Sept. 27	Treated with cholesterol.	9.573	4.15
2	Aug. 13	Oct. 7	Treated with cholesterol.	6.165	4.41
3	Aug. 13	Oct. 14	Treated with cholesterol.	18.563	3.97
4	Aug. 13	Oct. 17	Treated with cholesterol.	15.663	3.97
5	Aug. 13	Oct. 19	Treated with cholesterol.	37.113	3.75
6	Sept. 20	Oct. 21	Not treated. Two tumors weighed together.	1.000	2.6
7	Aug. 13	Oct. 23	Not treated. Tumor apparently disappearing.	1.288	9.7
8	Aug. 13	Oct. 27	Treated with cholesterol. Tumor apparently disappearing.	1.058	8.76
9	Sept. 20	Oct. 30	Not treated. Tumor young but already soft at center.	3.168	4.12
10	May 26	Oct. 31	Not treated.	Almost as large as rat	{ *2.35 †8.86
11	May 26	Nov. 18	Not treated.	Extremely large	{ *2.25 †5.71
12	Oct. 25	Nov. 25	Not treated. Three small tumors weighed together.	1.322	2.47

\* Portions of the outside or growing part alone were taken.

† Portions of the inside alone were taken.

The tumors in the first nine rats were of the eighth generation, in numbers 10 and 11 of the seventh generation, and in 12, of the ninth generation.

In practically all cases where the whole tumor could not be analyzed, the tumor was ground and carefully mixed to obtain a representative sample. In rats numbers 10 and 11, however, this was not done for obvious reasons. The determinations of cholesterol were made by the method of Autenrieth and Funk,<sup>3</sup> using ether for the extractions, and a Duboscq colorimeter.

<sup>3</sup> *Münch. med. Wochenschr.*, lx, p. 1243, 1913.

## THE DETERMINATION OF CREATINE IN MUSCLE.

By LOUIS BAUMANN.

(From the Chemical Research Laboratory, Medical Department, State University of Iowa, Iowa City.)

(Received for publication, December 14, 1913.)

A number of methods for the determination of the sum of creatine and creatinine in muscle may be found in the literature, many of which are tedious and time-consuming. In our hands the aqueous extraction method, recently employed by Myers and Fine<sup>1</sup> requires from one to one and a half laboratory days. The method described by Pekelharing and van Hoogenhuyze<sup>2</sup> is shorter but also entails the separate conversion of creatine into creatinine by the autoclave process of Benedict and Myers.<sup>3</sup>

The method in use in this laboratory for the past few months is relatively simple and is carried out in about one-quarter of the time required for the aqueous extraction method, while the results are, within the limits of the experimental error, identical with those obtained by either of the above-mentioned methods.

The proposed method is carried out as follows: Fifty grams of hashed muscle are weighed into a round-bottom, short-necked Jena flask; to this 125 cc. of 5 N sulphuric acid, and a few chips of unglazed porcelain are added and the whole boiled for three hours under a reflux condenser. At the end of this time the muscle is disintegrated. The solution is now filtered quantitatively through a 15-em. filter paper into a 250-cc. volumetric flask,<sup>4</sup> the residue (less than 2 grams of dry material) is washed thoroughly with distilled water, the fluid is cooled and the flask filled to the mark. Twenty cubic centimeters of the claret-colored extract are pipetted into a small porcelain dish (8.5 em. in diameter) and 18 cc. of

<sup>1</sup> This *Journal*, xiv, p. 9, 1913.

<sup>2</sup> *Zeitschr. f. physiol. Chem.*, lxiv, p. 262, 1910.

<sup>3</sup> *Amer. Journ. of Physiol.*, xviii, p. 397, 1907.

<sup>4</sup> The volume of the sulphuric acid and the dilution are proportional to the weight of muscle used for analysis.

10 per cent sodium hydrate are added while stirring.<sup>5</sup> The partially neutralized fluid is then evaporated on the water bath to about 10 cc. and transferred quantitatively to a 50-cc. volumetric flask containing 30 cc. of saturated aqueous picric acid solution (the solid particles adhering to the sides of the dish are of no consequence provided the washing has been thorough). The flask is filled to the mark with distilled water when its contents have reached the proper temperature, shaken vigorously, then filtered through a dry filter-paper. To 25 cc. of the clear filtrate 6 cc. of the 10 per cent sodium hydrate are added and the creatinine determined colorimetrically according to Folin, allowing ten minutes for the color to develop. The standard is a creatinine solution containing about 7 mgm. of creatinine per 10 cc. of solution. It may easily be prepared by heating pure anhydrous creatine (about 80 mgm.) with 50 cc. of 5 N sulphuric acid for three hours under a reflux condenser, then diluting at once in a volumetric flask to 100 cc. The titer of this solution remains constant for months.

#### EXPERIMENTAL.

(1) Creatine prepared according to the unpublished methods of S. R. Benedict<sup>6</sup> was recrystallized until it was creatinine-free, then rendered anhydrous by heating *in vacuo* at 56° over  $P_2O_5$  for two to three hours; 0.0520 gram of this substance when subjected to a Kjeldahl analysis required 11.85 cc. of  $\frac{N}{10}$  sulphuric acid, equivalent to 0.0166 gram of N. Found, 31.90 per cent of N. Calculated for creatine, 32.06 per cent.

(2) 0.1605 gram of this pure creatine was boiled for three hours under a reflux condenser with 75 cc. of 5 N sulphuric acid, then cooled at once and transferred to a 150-cc. volumetric flask and diluted to the mark; 10 cc. of this solution were analyzed for creatinine according to Folin's colorimetric method, using 8 mm. of  $\frac{N}{2}$  potassium bichromate solution as a standard. The average of seven readings was 8.77 mm. Found, 9.25 mgm. Calculated, 9.22 mgm. of creatinine.

(3) 0.1161 gram of analytically pure creatine was heated for three hours with 50 cc. of 5 N sulphuric acid under a reflux condenser, then diluted at once to 100 cc.; 10 cc. of the solution were used for the determination. Average of seven readings, 8.14 mm. Found, 9.95 mgm. Calculated, 10.00 mgm. of creatinine.

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<sup>5</sup> If  $\frac{N}{2}$  potassium bichromate solution is to be used as a standard, 25 cc. of the extract and 23 cc. of the alkali are used. The other steps remain unchanged.

<sup>6</sup> I take this occasion to thank Prof. S. R. Benedict for his kindness in sending to me his unpublished manuscripts.

(4) 0.1216 gram of pure creatine treated as above and then diluted to 100 cc.; 10 cc. of the solution were used for the determination. Average of seven readings 7.6 mm. Found, 10.66 mgm. Calculated, 10.74 mgm. of creatinine.

(5) 0.1407 gram of analytically pure creatine treated as above and then diluted to 150 cc.; 10 cc. of this solution were used for the determination. Average of seven readings, 10.02 mm. Found, 8.08 mgm. Calculated, 8.08 mgm. of creatinine.

(6) Twelve different samples of muscle, including ten of beef and two of rabbit were analyzed, both according to the method as described by Myers and Fine<sup>7</sup> and according to the method proposed, with the following results:

*Creatine in fresh muscle.*

	Aqueous extract method per cent	Proposed method per cent
	0.350	0.355
	0.376	0.368
	0.381	0.385
	0.395	0.398
Beef.....	0.443	0.440
	0.456	0.453
	0.463	0.467
	0.363	0.364
	0.411	0.407
	0.368	0.375
Rabbit.....	0.521	0.517
	0.532	0.528

Two samples of beef were analyzed according to the method of Pekelharig and van Hoogenhuyze<sup>8</sup> and compared with the newer process.

	Pekelharig and van Hoogenhuyze's method per cent	Proposed method per cent
Beef.....	0.431	0.427
	0.407	0.404

SUMMARY.

A method for the determination of the sum of creatine and creatinine in muscle is proposed, which is simple, relatively rapid, and accurate. The only reagent required besides the picric acid and alkali used in Folin's colorimetric determination is 5 N sulphuric acid.

We are beginning to apply this method to the determination of the sum of creatine and creatinine in blood and tissues.

<sup>7</sup> *Loc. cit.*

<sup>8</sup> *Loc. cit.*



## THE ABSENCE OF SUGAR IN THE URINE AFTER PANCREATECTOMY IN PREGNANT BITCHES NEAR TERM.

By A. J. CARLSON, J. S. ORR AND W. S. JONES.

(From the Hull Physiological Laboratory of the University of Chicago.)

(Received for publication, December 15, 1913.)

Experiments were reported two years ago showing that complete pancreatectomy in pregnant bitches near term does not result in diabetes as long as the fetuses are alive and remain in the uterus.<sup>1</sup> The absence of pancreatic diabetes was interpreted as showing the passage of the internal secretion of the fetal pancreas to the blood of the mother. The experiment has been repeated on one dog by Hedon,<sup>2</sup> without results. Hedon's dog did not urinate till the second day after the pancreatectomy, and the urine (210 cc.) contained 4.5 grams of glucose. On the third day there was a discharge of bloody mucus from the vagina, and the dog died of peritonitis on the fourth day. The pups were, of course, dead. As the weight of the pups averaged only 81 grams (the mother being a large dog, weighing 16 kgm.) it is clear that the pregnancy was not within two weeks of term. The peritonitis and the bloody discharge from the vagina indicate that the fetuses had died, probably soon after the operation. This experiment of Hedon's therefore proves or disproves nothing. Allen has repeated our experiment (with modification) on one dog.<sup>3</sup> This investigator extirpated nine-tenths of the pancreas in a pregnant bitch near term. No sugar appeared in the urine. One-tenth of the pancreas does not suffice to prevent diabetes in non-pregnant dogs. Allen's dog died on the seventh day after the pancreatectomy. There was no peritonitis, or other evidence of infection to account for the death. In view of our own results

<sup>1</sup> Carlson and Drennan: *Amer. Journ. of Physiol.*, xxviii, p. 391, 1911.

<sup>2</sup> Hedon: *Arch. internat. de physiol.*, xiii, p. 6, 1913.

<sup>3</sup> Allen: *Glycosuria and Diabetes*, Boston, 1913.

we think that Allen's dog died from toxemia in consequence of death of the fetuses, the uterus not being in condition to expel them.

Our first report contained in reality only one satisfactory and conclusive experiment. It seemed desirable to secure a greater number of satisfactory experiments so as to establish the fact securely before endeavoring to work out the mechanism of this control of pancreatic diabetes. It is so difficult to make a complete pancreatectomy in pregnant animals near term without causing abortion, death of the fetuses, or death of the mother from "shock," that we anticipate that many workers who repeat the experiment will have nothing but failures in the beginning.

Complete pancreatectomy was made in fourteen pregnant bitches in the spring of 1913. More than half of these experiments were failures, through abortion, death of the fetuses (probably from the anesthetic), or death of the mother from shock. Two of the operated bitches proved to be in early pregnancy. These two ran a typical course of pancreatic diabetes. Both dogs aborted (fetuses dead) on the sixth day after the operation. In no case did we succeed if the bitch was under anesthesia longer than forty-five minutes for the pancreas operation. But even the completion of the pancreatectomy in less than forty-five minutes and with the greatest care does not always assure success, owing probably to varying resistance of the animals. The experiments that were wholly or partly successful are summarized in Table I.

Dog 1 showed a light glycosuria for two days after the pancreatectomy. Then the pups were born, and that event was followed by the typical pancreatic diabetes. Dog 5 showed no trace of sugar in the urine for four days after removal of the pancreas, but *the typical pancreatic diabetes developed on the day of the birth of the pups*. These two dogs furnish the same clear-cut evidence as did Dog 5 in our earlier work. The results on Dogs 2-4 are more ambiguous. To be sure, these dogs showed no sugar in the urine for the three to four days after the pancreas extirpation, but the fact that the dogs died in depression or convulsion at the end of that time makes it possible that absence of glucose in the urine was due to some change in the kidneys leading to intoxication or eclampsia. This explanation is, however, rendered improbable by the copious secretion of urine.

It can therefore be considered as well established that late pregnancy (two to three weeks of term) prevents the appearance of sugar in the urine after complete pancreatectomy as long as

TABLE I.

DOG	DATE	URINE	GLU- COSE	GLUCOSE	REMARKS
		cc.	percent	grams	
1	May 10.....				Pancreatectomy.
	May 11.....	65	0.91	0.5915	
	May 12.....	200	1.3	2.6	
	May 13.....	390	3.2	12.58	{ Abortion from 10 a.m. to 3 p.m.: 6 pups alive and well developed.
	May 14.....	440	4.9	21.208	
	May 15.....	275	5.15	14.10	
	May 16.....	525	2.08	10.92	Experiment discontinued.
2	May 13.....				Pancreatectomy.
	May 14.....	205	0.2	0.4	
	May 15.....	485			
	May 16.....	200			{ Much depressed; discharge from vagina; Caesarian section 10 a.m.: pups dead; dog died in afternoon, probably due to toxemia.
	May 17.....				Pancreatectomy 11 a.m.
3	May 18.....	100			
	May 19.....	575			
	May 20.....	325			{ Dog found dead; no infection; advanced pregnancy. Pancreas removed completely.
	June 3.....				Pancreatectomy.
4	June 4.....	250			
	June 5.....	350			{ Dog died in convulsion; 10 pups well matured. No peritonitis.
	June 4.....				Pancreatectomy.
5	June 5.....	150			
	June 6.....	310			
	June 7.....				
	8.00 a.m....	380			{ 8 a.m.-1 p.m., 8 live, well developed pups born.
	10.30 a.m....	100	7.16	7.16	
	1.30 p.m....	150	3.72	5.58	
	June 8 8.00 a.m....	380	1.29	4.00	Experiment discontinued.

the fetuses remain alive and in the uterus. But despite this absence of the characteristic diabetes, the pancreatectomy leads to changes in the mother that are incompatible with the pregnancy itself. Consequently the fetal pancreas is not capable of complete substitution for the maternal pancreas. The birth of the pups was premature both in Dog 1 and Dog 5 and we have already stated that the two dogs that were depancreatized in early pregnancy aborted their immature and dead fetuses a few days after the operation. Complete pancreatectomy is therefore incompatible with pregnancy at all its stages, either through toxemia of the blood or malnutrition of placenta and fetus. It is highly probable that absolute diabetes is incompatible with ovulation and the implantation of the ovum in the uterus.

In our first communication we interpreted our results as due to the passage of the internal secretion of the fetal pancreas to the blood and tissues of the mother. Macleod and Pearce point out that the facts can be equally well explained on the "detoxication theory" of pancreas activity,<sup>4</sup> the hypothetical toxins passing from the maternal to the fetal blood and are taken care of by the fetal pancreas. This is a possibility. But the detoxication theory remains a theory, while the internal secretion theory is being gradually translated into fact. There is a third possibility. The excess sugar may pass into the blood of the fetuses to be stored or oxidized.

We have been impressed by the great disturbance evidenced in the dogs deprived of the pancreas near the end of the pregnancy despite the fact that sugar does not appear in the urine. The dogs lose weight and show depression and weakness, and the pregnancy is terminated either by death of the fetuses or by abortion. Can it be that the glycosuria of diabetes may be absent in the presence of fatal disturbance of metabolism induced by complete removal of the pancreas? If it was possible to prevent abortion or death of the fetuses for two or three weeks after the pancreatectomy we believe that the pregnant dogs would show the typical decline of pancreatic diabetes without at any time having sugar in the urine. Would that be pancreatic diabetes? Or must we look for other guides than glycosuria in the ultimate analysis of diabetes?

<sup>4</sup> Macleod and Pearce: *Amer. Journ. of Physiol.*, xxxii, p. 184, 1913.

# THE INFLUENCE OF PREGNANCY AND CASTRATION ON THE IODINE AND PHOSPHORUS METABOLISM OF THE THYROID GLAND.<sup>1</sup>

By FREDERIC FENGER.

(From the Research Laboratory in Organotherapeutics of Armour and Company.)

(Received for publication, December 17, 1913.)

It has been demonstrated by the investigations of Seidell and Fenger<sup>1</sup> that there exists a distinct seasonal variation in the iodine content of the thyroid gland from domestic animals. For the purpose of establishing more definite conclusions regarding the probable influence of pregnancy and of castration upon the thyroid activity, the present investigation was conducted on composite lots of glands from bulls, steers, pregnant and non-pregnant cows respectively. The months of October and November were chosen for collecting the raw material, because cattle on the whole are in excellent condition at this particular time and practically no large or goitrous glands are encountered.

During the fall season, when the iodine content of the thyroid gland is high, a pungent garlic-like odor closely resembling that of oxidizing phosphorus is noticed not only in the thyroid gland proper, but also in the abattoir, when the throat of the animal is opened. This is explained by the fact that as a rule at least one of the lobes of the thyroid is cut in two. It is very likely that the iodine interacts with the phosphates in the thyroid gland producing an organic complex which possesses the characteristic garlic-like odor of certain phosphorus compounds. The fact that the odor is noticeable before death when the thyroid is cut open would indicate that the reaction actually takes place during life. That it is the iodine in thyroid combination, and not the organic phosphates, which is the positive and activating factor in producing this reaction is equally evident because the odor is pronounced

<sup>1</sup> A. Seidell and F. Fenger: *This Journal*, xiii, p. 517, 1913.

only when the iodine content is high and has never been detected in normal sized glands low in iodine or in enlarged and diseased thyroids. Large glands generally show a high amount of phosphates and a low percentage of iodine.

That a part of the phosphorus in the thyroid gland is closely associated with the iodine complex is shown by the following experiment, conducted on a lot of several hundred well trimmed, minced and mixed beef glands. A portion of the fresh glands was desiccated and freed from fat in the usual way, while the other portion was macerated with physiological salt solution for eighteen hours. The mixture was then filtered and the filtrate precipitated at body temperature with very dilute acetic acid. The precipitate was washed by decantation and on the filter with acid water and then redissolved in water containing a small but sufficient amount of sodium hydroxide. The precipitation was repeated and the final precipitate washed with acidulated water, dried at low temperature and powdered. The original desiccated fat-free glands showed 0.39 per cent of iodine and 0.99 per cent  $P_2O_5$ , while the thyroid proteins contained 0.61 per cent of iodine and 0.26 per cent of  $P_2O_5$ . The high percentage of iodine and the low percentage of phosphorus in the precipitated proteins indicate the existence of iodine in thyroid combination both free and combined with phosphorus while the high percentage of phosphorus in the glands themselves shows the presence of organic phosphates uncombined with iodine.

The distinction between pregnancy and non-pregnancy was determined by a thorough examination of the uterus of each individual female animal. Systematic discrimination was exercised in selecting the glands from pregnant cows with the viewpoint of having the final lot represent uniformly the entire period of gestation. Non-pregnant cows include about 50 per cent of virgin animals (heifers), the balance being cows which have given birth to one or more calves. Cases where the uteri were found to be large or extended from recent calving were excluded from the present investigation.

In order that the analytical data should be truly representative of actual existing conditions, special precautions were taken to collect an equal or at least proportional number of glands from the different kinds of animals on the same day. Collections were

made twice a week during the entire period. The number of animals used for this investigation was as follows: Bulls, 470; steers, 1068; pregnant cows, 1123; non-pregnant cows, 1021. It was impossible to secure the same number of bulls as of the other animals. The number, however, is sufficient to eliminate the individual variations of the therapeutic activity of the thyroid gland and the analytical data are, therefore, comparative.

The total number and average weight of the various live animals as well as the maximum, minimum and average weight of the fresh, trimmed single lobes and the entire glands, are given in Table 1, together with the moisture and fat content of the fresh glands and yield of the desiccated fat-free material. No distinction was made between the right and left lobes and the weight of the entire gland was obtained by multiplying by two the average weight of the single lobes.

The glands were removed from the oesophagus of the various animals shortly after slaughtering, and while still retaining the animal heat, carefully freed from connective and other adherent tissues and as far as possible from fat, weighed and stored at freezing temperature until the entire number had been collected. The four lots of glands were then finely minced and representative samples of 2000 grams each dried on agate-ware trays at a temperature not exceeding 60°C.; they were then freed from fat by petroleum ether in Soxhlet extractors. The losses of moisture and fat on the four lots of fresh glands were recorded and the dried fat-free material powdered in a tube mill to pass a 60-mesh sieve. On these samples moisture, ash, total phosphoric acid and iodine were determined.

The various determinations were made in duplicate on two separate sets of samples, drawn on two different days from the main bulk of the powdered glands, and the figures given in Table 2 are the average results of these four determinations. The iodine was estimated by the Hunter method<sup>2</sup> and the total phosphoric acid determined according to the official volumetric method.<sup>3</sup> The determinations of moisture were made on 2.5-gram samples by placing the material in shallow porcelain dishes and drying at

<sup>2</sup> A. Hunter: *This Journal*, vii, p. 321, 1910.

<sup>3</sup> Bulletin 107 (revised), Bureau of Chemistry, 1908.

100°C. to constant weight. This required about five hours. The ash was estimated on 1-gram samples weighed into porcelain crucibles of 15 cc. capacity and placed in a muffle furnace previously heated to dull redness. This temperature permits complete combustion of the organic material and at the same time prevents fusion of the ash. The complete ignition required about two hours.

Table 3 gives the weights of phosphorus and iodine in the whole fresh glands together with the amounts of fresh thyroid tissue, phosphorus and iodine per 100 pounds of body weight of the various animals.

The tabulated figures show very plainly that female animals contain more thyroid tissue and considerable more iodine per unit of body weight than the male animals.

Castrated male animals show about 80 per cent thyroid tissue and a little below 90 per cent of iodine in thyroid combination per unit of body weight when compared with female animals.

There is practically no difference in the size and iodine content of thyroid glands from pregnant and non-pregnant animals.

It has been shown in a previous communication that the iodine content from female fetal glands is higher than that from male glands.<sup>4</sup> It has also been demonstrated that the fetal thyroid gland contains iodine as early as three months after conception, and that it is therapeutically active and contains more iodine per unit of body weight during the last three months of gestation than the glands from adult animals.<sup>5</sup> When all these facts are taken into consideration it seems very evident that thyroid tissues in general, whether fetal or adult, possess a pronounced and individual selective affinity for iodine and that the fetal tissue performs its function throughout gestation absolutely independently of the maternal tissue, both, of course, receiving their respective supplies of iodine from the same source.

The phosphorus content is low and fairly uniform in all four instances, simply indicating normal physiological activity of the gland.

<sup>4</sup> F. Fenger: *This Journal*, xiv, p. 397, 1913.

<sup>5</sup> *Ibid.*

TABLE 1.  
*Fresh glands.*

	TOTAL NUMBER OF ANIMALS	AVERAGE WEIGHT OF ANIMALS	MAXIMUM WEIGHT OF SINGLE LOBE	MINIMUM WEIGHT OF SINGLE LOBE	AVERAGE WEIGHT OF SINGLE LOBE	AVERAGE WEIGHT OF WHOLE GLAND	MOISTURE IN FRESH GLANDS	FAT IN FRESH GLANDS	YIELD OF DESIC- CATED FAT-FREE GLANDS
		lbs.	grams	grams	grams	grams	per cent	per cent	per cent
Bulls.....	470	1400	50.7	8.0	15.0	30.0	69.0	6.9	24.1
Steers.....	1068	1200	28.0	4.5	11.5	23.0	64.0	10.7	25.3
Pregnant cows.	1123	1000	40.0	6.0	12.2	24.4	68.5	9.0	22.5
Non-pregnant cows and heifers.....	1021	925	33.0	5.0	11.3	22.6	65.0	9.3	22.7

TABLE 2.  
*Desiccated glands.*

	MOISTURE LOST AT 100°C.	ASH	PHOSPHORUS	IODINE	ASH CALCULATED TO DRY BASIS	PHOSPHORUS CALCULATED TO DRY BASIS	IODINE CALCULATED TO DRY BASIS
	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Bulls.....	4.25	3.99	0.424	0.268	4.17	0.443	0.279
Steers.....	3.04	4.00	0.412	0.339	4.12	0.425	0.350
Pregnant cows.....	3.74	4.20	0.384	0.337	4.36	0.399	0.350
Non-pregnant cows and heifers.....	3.99	4.11	0.400	0.331	4.28	0.417	0.345

TABLE 3.

	PHOSPHORUS CALCULATED IN WHOLE FRESH GLANDS	IODINE CALCULATED IN WHOLE FRESH GLANDS	FRESH THYROID TISSUE CALCULATED PER 100 POUNDS OF BODY WEIGHT	PHOSPHORUS CALCULATED PER 100 POUNDS OF BODY WEIGHT	IODINE CALCULATED PER 100 POUNDS OF BODY WEIGHT
	mgms.	mgms.	grams	mgms.	mgms.
Bulls.....	30.66	19.38	2.14	2.19	1.38
Steers.....	23.97	19.73	1.92	2.00	1.64
Pregnant cows.....	21.08	18.50	2.44	2.11	1.85
Non-pregnant cows and heifers.....	20.52	16.98	2.44	2.22	1.84

## SUMMARY.

Female animals contain more thyroid tissue and iodine in thyroid combination per unit of body weight than male animals, indicating greater functional activity of the female glands. This is analogous to the conditions existing in the fetus during intra-uterine life.

No apparent difference in size and physiological activity exists between glands from pregnant and non-pregnant female animals, and the increased iodine metabolism of the fetal glands, both male and female, seems therefore to be independent of the maternal glands, having in common only the source of the iodine.

Male animals contain a little less thyroid tissue per unit of body weight than the females and only three-fourths the amount of iodine in thyroid combination.

Castrated males contain less thyroid tissue than either uncastrated males or females, but the iodine content per unit of body weight is about half way between the uncastrated male and female animals.

The phosphorus content of the thyroid gland seems to be fairly uniform in all four cases and should only be considered an indication of normal physiological activity.

The garlic-like odor observed in all four lots of glands is due directly to the comparatively large amounts of iodine present and furnishes evidence of a chemical reaction specific to the thyroid and resulting in an organic complex possessing the characteristic odor of oxidizing phosphorus. The odor was observed before the death of the animals, indicating that the reaction actually takes place during life.

# THE RESOLUTION OF INACTIVE URAMIDO-ACIDS AND HYDANTOINS INTO ACTIVE COMPONENTS, AND THEIR CONVERSION INTO AMINO-ACIDS.

## I. $\beta$ -PHENYL- $\alpha$ -URAMIDOPROPIONIC ACID, BENZYLHYDANTOIN AND PHENYLALANINE.

By H. D. DAKIN AND H. W. DUDLEY.

(From the Herter Laboratory, New York.)

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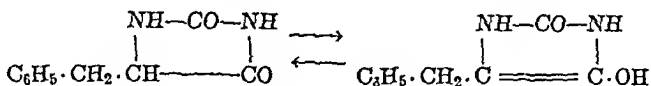
The ordinary  $\alpha$ -amino-acids are so feebly acidic that their resolution into optically active components by means of their salts formed with active bases cannot be effected. Fischer and his co-workers have shown, however, that the acyl derivatives, especially the formyl, acetyl and benzoyl compounds of amino-acids form well crystallized salts with alkaloids, from which active amino-acids may be recovered by hydrolysis.

In connection with the use of uramido-acids for the identification of certain amino-acids, it was desirable for us to obtain active  $\beta$ -phenyl- $\alpha$ -uramidopropionic acid. We have now found that by the use of a suitable active base, inactive  $\beta$ -phenyl- $\alpha$ -uramidopropionic acid, obtained by the action of potassium cyanate upon phenylalanine, may be readily resolved into its active components.

By preparing the strychnine salts of the uramido-acid and crystallizing from a mixture of methyl alcohol and acetone, the resolution may be effected with unusual ease, so that it is possible to obtain 70-80 per cent of the theoretical amount of both active forms. This method of preparation of the active forms proved to be much simpler than effecting a prior separation of phenylalanine into active components and then converting them into the uramido-acids by the action of cyanate.

On heating the active uramido-acids with dilute hydrochloric acid, active benzylhydantoins with an opposite sign of rotation were obtained. The active benzylhydantoins on dissolving in

dilute ammonia or sodium hydroxide underwent racemization precisely in the same fashion as has been previously observed with other active hydantoins.<sup>1</sup> Racemization in these cases is no doubt due to keto-enol tautomerism involving loss of asymmetry.



Since either active hydantoin, and hence uramido-acid also, may be readily racemized in this fashion and subsequently reconverted into the inactive uramido-acid, it is possible theoretically to convert the whole of the original uramido-acid into either active form. While this cannot be effected practically in a strictly quantitative fashion, it is nevertheless true that since the uramido-acids and hydantoins are such well crystallized compounds undergoing ready interconversion it is possible to approximate this result surprisingly closely.

Since uramido-acids may be reconverted into amino-acids by hydrolysis with hydriodic acid or with barium hydroxide, it appeared probable that our observations upon the resolution of inactive uramido-acids might be made the basis of a convenient method of resolving amino-acids. Unfortunately hydrolysis of either active  $\beta$ -phenyl- $\alpha$ -uramidopropionic acid or benzylhydantoin with either hydriodic acid or baryta, was accompanied by almost complete racemization. Our results cannot therefore be employed for the preparation of active phenylalanines. Efforts to find more suitable agents for hydrolysis, e.g., sulphuric or phosphoric acids, were fruitless.

On hydrolyzing active  $\beta$ -phenyl- $\alpha$ -uramidopropionic acid incompletely with hydriodic acid, we obtained a mixture of inactive phenylalanine and inactive benzylhydantoin. It is clear, therefore, that racemization takes place prior to amino-acid formation. The fact that boiling hydriodic acid effects racemization so readily, makes it appear probable that active hydantoins prepared by the action of acids on pure active uramido-acids are partially racemized. Our results with benzylhydantoin indicate that our substances were not optically homogeneous since starting with

<sup>1</sup> Dakin: *Amer. Chem. Journ.*, xlv, p. 48, 1910.

pure uramido-acid of constant rotation, we obtained benzylhydantoin of varying optical activity.

The action of hydrochloric acid in effecting the racemization of benzylhydantoin, was shown by the fact that a sample of the hydantoin with a specific rotation in 50 per cent alcohol of  $-79.7^\circ$ , after warming for two hours on the water bath with concentrated hydrochloric acid diluted with an equal volume of water, had a rotation of only  $-56.6^\circ$ . It appears probable that the racemization of hydantoins by acids is due to tautomeric change involving keto-enol isomerism, as is the case with the action of alkali already referred to.

In the preparation of active hydantoins from uramido-acids, it is clearly of importance that the boiling with acid be not prolonged beyond the time necessary for the conversion and that as dilute acid as possible be employed.

On hydrolyzing active  $\beta$ -phenyl- $\alpha$ -uramidopropionic acid with barium hydroxide, although racemization was almost complete, it was just possible to show that the dextro uramido-acid gave laevo phenylalanine and vice versa. This change in the sign of rotation on passing from the uramido-acid to phenylalanine or benzylhydantoin was confirmed by taking *l*-phenylalanine and converting it into *d*- $\beta$ -phenyl- $\alpha$ -uramidopropionic acid by means of potassium cyanate and then obtaining *l*-benzylhydantoin by the action of dilute acid.

Mention may be made of the fact that inactive  $\beta$ -phenyl- $\alpha$ -uramidopropionic acid was first obtained by one of us from the urine of cats which had received injections of phenylalanine and it was subsequently obtained synthetically.<sup>2</sup> The dextro modification has been described recently by Embden and Schmitz<sup>3</sup> and was used by them for the identification of *l*-phenylalanine obtained on perfusion of the liver with ammonium phenylpyruvate. The melting point is given as  $179-180^\circ$ , but we believe this to be considerably too low as our compound melts at  $195-196^\circ$ . It is not impossible that Embden and Schmitz's compound was partially racemized owing to its method of preparation—by boiling the amino-acid solution with sodium hydroxide and urea. This ap-

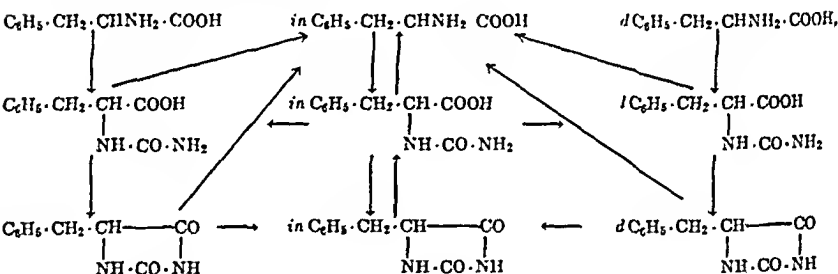
<sup>2</sup> This *Journal*, vi, p. 235, 1909.

<sup>3</sup> *Biochem. Zeitschr.*, xxxviii, p. 393, 1912.

appears possible since our own observations have shown that extensive racemizations follow prolonged boiling of the uramido-acid with barium hydroxide. Another possibility is that partial conversion of the uramido-acid into benzylhydantoin occurred in the course of its extraction from acid solution. The melting points of these compounds are readily depressed by small amounts of optical isomers or other impurity. We do not suggest, however, that the observed differences in melting point in any way invalidate the identification of phenylalanine by Embden and Schmitz.

Inactive benzylhydantoin was first obtained by Wheeler and Hoffman<sup>4</sup> and is described as melting at 190°. We find, on repeated crystallization from water, that the melting point rises to 194.5–195.5°.

The various reactions previously referred to may be shown graphically as follows:



#### EXPERIMENTAL.

##### *The resolution of inactive β-phenyl-α-uramidopropionic acid.*

The inactive uramido-acid was obtained as previously described<sup>5</sup> by evaporating a solution of equal weights of phenylalanine and potassium cyanate in ten parts of water. On acidifying the solution with hydrochloric acid, the uramido-acid is precipitated in a pure condition. The yield is 90 per cent of the theoretical amount. It may be recrystallized from boiling water and crystallizes in colorless prisms melting with effervescence at 188–190°.

The resolution of the inactive acid may be effected as follows:

<sup>4</sup> *Amer. Chem. Journ.*, xlv, p. 368, 1911.

<sup>5</sup> *This Journal*, vi, p. 241, 1909.

The uramido-acid (5.6 grams) is mixed with finely powdered strychnine (9.1 grams) and boiled with 30 cc. of methyl alcohol until only the slight excess of strychnine remains undissolved. The hot solution is then filtered using 5 cc. more of methyl alcohol for washing. Acetone (50 cc.) is then added to the clear filtrate. After a short time glistening crystals of the laevo strychnine salt begin to separate and after standing for twenty-four hours in a cool place, a little over 80 per cent of the theoretical amount of salt will have separated. The crystals are filtered off and washed with acetone. The mother liquor on concentrating to 25 cc. and then adding 50 cc. more of acetone, deposits on standing a second crop of the same salt amounting to 10-15 per cent of the theoretical amount.

The strychnine salt crystallizes from methyl alcohol and acetone in glistening plates often arranged in the form of sheaves. It is only sparingly soluble in water, methyl alcohol or chloroform and may be recrystallized from a mixture of methyl alcohol and water. Prolonged boiling with anhydrous methyl alcohol tends to produce a less soluble modification of the salt. It is very slightly soluble in acetone.

Strychnine *l*- $\beta$ -phenyl- $\alpha$ -uramidopropionate melts at 225.5-226°.

Its rotation was observed in methyl alcohol solution.  $c = 2.0$ ;  $l = 2.2$  dm.;  $\alpha = -1.45^\circ$ .

$$[\alpha]_D = -33.0^\circ.$$

0.1000 gram gave 0.0102 gram N = 10.2 per cent.

$C_{21}H_{21}N_4O_6$  requires 10.3 per cent N.

The melting point and rotation were unchanged on recrystallization.

Mention may be made of the fact that crystallization from chloroform of the strychnine salts of the inactive uramido-acid failed to effect any significant resolution. The substitution of quinine for strychnine also gave negative results.

*l*- $\beta$ -Phenyl- $\alpha$ -uramidopropionic acid. The active uramido-acid was obtained by dissolving the strychnine salt in a large quantity of boiling water and then adding an excess of ammonia. On cooling, the precipitated strychnine is filtered off and the ammonium salt of the uramido-acid evaporated almost to dryness on the water bath. A slight excess of hydrochloric acid is then added

and the precipitated uramido-acid filtered off and recrystallized from boiling water. The yield is excellent and the substance as first obtained is optically homogeneous. *l*- $\beta$ -Phenyl- $\alpha$ -uramidopropionic acid crystallizes from water in which it is sparingly soluble in clear prisms melting at 195–196°.

0.1070 gram gave 0.0144 gram N = 13.5 per cent.

$C_{10}H_{12}N_2O_4$  requires 13.5 per cent N.

The rotation was observed in 1 per cent solution in normal ammonia (average of four determinations).  $c = 1.0$ ;  $l = 2$ ;  $\alpha = 0.738^\circ$ .

$[\alpha]_D = -36.9^\circ$ .

The melting point and rotation are unchanged on recrystallization. It is slightly less soluble in water than its inactive isomer.

*d*- $\beta$ -Phenyl- $\alpha$ -uramidopropionic acid. The mother liquor from the strychnine salt of the *l*-uramido-acid was treated with excess of ammonia and diluted freely with water. The precipitated strychnine was filtered off, and the filtrate concentrated on the water bath to small volume. A little more strychnine separated out and was removed by filtration. The filtrate on acidifying with hydrochloric acid gives a large precipitate of the crude dextro uramido-acid. After a single recrystallization from boiling water, using a little charcoal, more than 80 per cent of the theoretical amount is obtained in an almost pure condition. The specific rotation of the once recrystallized substance varied in three separate preparations, between +35.7 and +35.9. After one more crystallization the melting point and rotation are constant.

*d*- $\beta$ -Phenyl- $\alpha$ -uramidopropionic acid crystallizes from water in clear prisms resembling the *l* isomer, and melts at 195–196°:

0.1412 gram gave 0.0190 gram N = 13.5 per cent N.

$C_{10}H_{12}N_2O_4$  requires 13.5 per cent N.

The specific rotation was observed in 1 per cent solution in normal ammonia (average of four determinations).  $c = 1.0$ ;  $l = 2$ ;  $\alpha = +0.726^\circ$ .

$[\alpha]_D = +36.3^\circ$ .

*d*- $\beta$ -Phenyl- $\alpha$ -uramidopropionic acid was also obtained by warming *l*-phenylalanine with potassium cyanate in aqueous solution and subsequently acidifying with hydrochloric acid. The precipitated uramido-acid was then crystallized from water. The properties of the substance thus prepared were identical with those recorded above.

*Hydrolysis of d- and l-β-phenyl-α-uramidopropionic acids.* One-gram portions of the active uramido-acids were gently warmed with 5 cc. of hydriodic acid (sp. gr. 1.9) for ten hours. The solution was then evaporated *in vacuo* and filtered from a precipitate of benzylhydantoin (0.3 gram). The hydantoin in each case was found to be optically inactive. The filtrate from the hydantoin was made alkaline with ammonia and evaporated to small bulk. Crystals of phenylalanine were readily obtained (0.4 gram) and were washed with hot alcohol. The phenylalanine melted at about 260° and was optically inactive.

In another experiment, *l*-β-phenyl-α-uramidopropionic acid (1 gram) was boiled for ten hours with 5 grams of barium hydroxide and 25 cc. of water. The solution was freed from barium with sulphuric acid and on concentrating gave a good yield of phenylalanine. The amino-acid showed a faint but definite dextro-rotation. Racemization was almost complete.

*d-Benzylhydantoin.* This substance was readily obtained by boiling for two hours the corresponding *l*-uramido-acid with twenty times its weight of dilute hydrochloric acid (1:5). On cooling, the hydantoin separates out in crystals resembling the uramido-acid but having a lower melting point and an opposite sign of rotation. The yield is about 90 per cent of the theoretical amount. The substance as obtained by us did not materially change its rotation on repeated crystallization from water, but since we have shown that boiling hydrochloric acid effects some racemization of the hydantoin, our figures can only be regarded as minimal values.

*d-Benzylhydantoin* crystallizes from water in transparent prisms which become opaque on drying. It is sparingly soluble in cold water, easily soluble in hot water and moderately soluble in alcohol. It melts at 181–183° without decomposition.

0.1084 gram gave 0.0161 gram N = 14.8 per cent N.

$C_{10}H_{10}N_2O_2$  requires 14.7 per cent N.

The rotation was observed in 50 per cent alcohol.  $c = 0.503$ ;  $l = 2.0$ ;  $\alpha = +0.97^\circ$ .

$$[\alpha]_D = +96.4^\circ.$$

*Racemization of d-benzylhydantoin.* The rotation of *d*-benzylhydantoin was also observed in normal ammonia in 1 per cent solu-

tion. The initial rotation was  $+2.35^\circ$  in a 2 dm. tube corresponding to  $[\alpha]_D = +106.8^\circ$ . On standing at room temperature, the rotation fell to about half its original value in twenty hours and to one-tenth in seventy hours and after standing several days longer, the solution became entirely inactive. On acidifying the solution, inactive benzylhydantoin melting at  $194^\circ$  was readily obtained. The racemization of benzylhydantoin with alkali is precisely similar to the change previously recorded by us in the case of other hydantoins.

*l-Benzylhydantoin.* The laevo hydantoin was obtained in precisely the same fashion as the dextro isomer. The substance crystallized from water in clear prisms melting at  $181-183^\circ$  and showing slight softening at  $179^\circ$ .

0.1076 gram gave 0.0160 gram N = 14.8 per cent N.

$C_{10}H_{10}N_2O_2$  requires 14.7 per cent N.

The specific rotation observed under the same conditions as the *d* compound gave a value of  $-79.7^\circ$  and this was unchanged on recrystallization from water. As previously mentioned, this figure must be too low owing to partial racemization taking place during its preparation from the *d*-uramido-acid. On heating the hydantoin two hours on the water-bath with a little concentrated hydrochloric acid diluted with an equal volume of water, the rotation of the substance fell to  $-56.6^\circ$ . On prolonged heating with concentrated acid, racemization is complete.



## PREPARATIONS ADMINISTERED.

Before describing the methods and results of these experiments it is perhaps well to indicate briefly the source and degree of purity of each of the materials employed.

*Sodium nucleate.* This was the same preparation as was used in our earlier experiments, and contained 8.68 per cent of purine nitrogen.

*Guanine.* Commercial guanine (Grübler) was converted into the sulphate. From this the base was obtained pure by solution in alkali and precipitation with acetic acid. Its nitrogen content was 46.3 per cent; theory demands 46.35.

*Adenine.* This was prepared from tea, and purified over the picrate. The free base as finally injected contained only 51, instead of the expected 51.8, per cent of nitrogen. It contained therefore still some admixture, probably of guanine.

*Xanthine* was made from the crude guanine preparation by treatment with sodium nitrite and sulphuric acid. After purification it yielded 36.5 per cent of nitrogen; calculated 36.85.

*Hypoxanthine* was obtained in the usual way from meat extract. The product had to be purified over the picrate, from which the free base was isolated with a nitrogen content of 41.3 per cent; theoretical 41.17.

*Uric acid.* A Kahlbaum specimen was purified by thrice recrystallizing from water. It contained the theoretical amount (33.3 per cent) of nitrogen.

It will be seen that of the free purines all except adenine were administered in a high state of purity.

## METHODS OF ADMINISTRATION.

In two of the experiments uric acid was fed by the mouth, being dissolved in a small quantity of dilute sodium hydroxide solution and mixed with the morning milk. In all the others the substances were introduced by subcutaneous injection. We are well aware of the objections that have been urged against parenteral methods of administration.<sup>2</sup> Purines are compounds to which the organism is not always wholly indifferent. The sudden introduction at one time of a considerable quantity of, say, uric acid, is liable to result in such an increased destruction of protein as strongly suggests a toxic influence; and this may be further manifested by an accelerated breakdown of endogenous purines.

<sup>2</sup> Cf. Schittenhelm: *Zeitschr. f. physiol. Chem.*, lxii, p. 80, 1909; Schittenhelm and Seisser: *Zeitschr. f. exp. Path.*, vii, p. 116, 1909.

Moreover, when its path is an unnatural one, a considerable proportion of the purine introduced is likely to be eliminated by the kidneys without having entered the metabolic cycle at all, and especially without having been submitted to the potent action of the liver. In any inquiry in which the maintenance of normal ratios is important, this last objection would be serious and even fatal. But, when the problem is to balance income and output, it is a first essential that the substance studied be known to have actually entered the organism, and to have entered it as such. Certainty upon this point is to be gained only by avoiding the intestinal route altogether; for, even if we can assure ourselves that a given compound has been in reality absorbed, we cannot tell to what extent environment and bacterial action may have previously altered its constitution. From our point of view this consideration outweighed all others. Adopting therefore the plan of subcutaneous injection, we have endeavored to minimize its admitted dangers by using doses that were comparatively small; while on two occasions we attempted to apply this principle even more thoroughly by dividing the dose into several fractions, the administration of which was spread over great part of a day.

In the majority of experiments an anesthetic was employed. The monkey was first brought as rapidly as possible under the influence of chloroform, and insensibility was thereupon maintained with ether until the introduction of the solution was accomplished. Narcosis was generally established in less than a minute, and the whole operation never lasted more than ten. So brief a period of anesthesia seemed to us less likely to exert a modifying influence upon the purine output, than the strenuous struggle which we learnt to be inevitable if anesthetics were dispensed with.

#### METHODS OF ANALYSIS.

For *allantoin* throughout, and for *purines* up to period 67, we adhered to the methods of our earlier communication. For later periods the Krüger-Schmid process was so combined with the Folin-Macallum method<sup>3</sup> for uric acid as to give us the means of

<sup>3</sup> Folin and Macallum: this *Journal*, xiii, p. 363, 1912; see also Folin and Denis: *ibid.*, xiii, p. 469, 1912, and xiv, p. 95, 1913.

determining the latter separately from the bases. As the combination has proved useful on many other occasions, we here describe it in full.

The first copper-purine precipitate (from two-fifths of the period urine) is suspended in about 200 cc. of water, to which there is added about 1 cc. of concentrated hydrochloric acid. The mixture is vigorously boiled, whereupon the whole or greater part of the precipitate goes into solution. Removal of the copper is effected by treatment with hydrogen sulphide in the heat, and the excess of the latter is completely expelled by renewed boiling. Filtration under suction, and thorough washing of flask and filter result in a filtrate which is perfectly clear and nearly colorless. This is concentrated, if necessary, and made up to a convenient volume (in the present case 100 cc.), which must of course be sufficiently large to retain, when cool, the uric acid in solution. Of this an aliquot part (here 10 cc.) is utilized directly for the colorimetric determination of uric acid. In the remainder the residual uric acid is destroyed, and the bases determined, according to the regular Krüger-Schmid procedure.

This application of the colorimetric method can be recommended whenever it is desired to determine uric acid and bases in one sample of urine, and especially when the amount of uric acid present is minute. It has enabled us in the present instance to estimate that substance in urine where previously we could not even detect its presence. We are confident that it yields figures to be depended on. We have frequently taken occasion, with urines richer in uric acid than the monkey's, to compare its results with those of the regular method of isolation and nitrogen determination. A few representative data of this nature are submitted in Table I.

TABLE I.

ANIMAL	GRAMS URIC ACID NITROGEN DAILY	
	By Colorimeter	By Kjeldahl
Sheep.....	0.046	0.043
	0.028	0.030
	0.029	0.030
Man.....	0.179	0.180
	0.115	0.119
Raccoon.....	0.0049	0.0045
Opossum.....	0.0034	0.0038
Goat.....	0.037	0.037

We feel justified in the conclusion that the copper precipitate is not liable to contain chromogenic substances other than uric acid, and that the plan we adopted attains all the accuracy of which the copper method in itself is capable.

If the values for uric acid reported below (Table II) be examined, it will be seen that the quantity of monkey urine taken for analysis must have contained from 4 to 5 mgms. of uric acid. This is after all not so very little, and it appears at first sight rather surprising that it should not have been detected in the usual way in our earlier experiments. The reason we believe to be two-fold. In the first place it is exceedingly probable that a quantity of uric acid absolutely perhaps small, yet relatively considerable, was destroyed by the employment of alkaline sodium sulphide to decompose the copper precipitate; and secondly, it is quite certain that the solubility of uric acid is increased by the simultaneous presence of an excess of purine bases. Each of these sources of error was avoided in the present series; the one by carrying out the decomposition in an acid medium,<sup>4</sup> and the other by utilizing the colorimetric method. It is, by the way, one very real advantage of the latter, that it renders one altogether independent of that "correction for solubility" which is a weak point in most other methods for uric acid.

#### EXPERIMENTS AND ANALYTICAL RESULTS.

The data which we have now to present and discuss will be found in Table II, the arrangement of which is determined not by the logical connection, but by the actual time-sequence of our experiments. It would be of little advantage to give precise dates, but it may be worth recording that the first period (45) of this table is separated from the last (42) of our earlier paper by an interval of four days; that about a month elapsed between 49 and 60, more than ten months between 67 and 78, eight days between 93 and 96, and five weeks between 102 and 105.

From period 45 to 49 the daily diet consisted of 150 cc. of milk, 150 grams of bananas, and 20 grams of peanuts; in all subsequent periods the allowance of milk was reduced to 125 cc. As a matter of fact, however, the monkey's appetite, quite independently of the injections, was very capricious, and it was rather the exception than the rule for it to consume the entire ration. For this

<sup>4</sup>This modification of the original procedure was first suggested, we believe, by Schittenhelm; see *Abderhalden's Handbuch der biochemischen Arbeitsmethoden*, III, p. 886, footnote.

TABLE II.

PERIOD	OUTPUT OF 48 HOURS				PERCENT- AGE OF TOTAL PURINE- ALLANTOIN N AS			URICOLYTIC INDEX	REMARKS
	Total N	Allantoin N	Uric Acid N	Purine Base N	Allantoin	Uric Acid	Purine Bases		
	grams	mgms.	mgms.	mgms.					
45	3.60	29	—	11					{ First uric acid injection: 0.200 gram.
46	2.81	44	15	19					
47	2.55	37	6	14					Weight: 4800 grams.
48	2.72	41	5	10					
49	2.15	32	4	11					Weight: 4700 grams. { First uric acid feeding: 0.103 gram.
60	2.70	29	—	13					
61	2.77	30	+	12					{ Second uric acid feeding: 0.198 gram.
62	2.91	28	—	12					
63	2.75	28	—	10					Weight: 4780 grams.
64	2.70	28	—	10					
65	2.92	30	5	10					{ Second uric acid injection: 0.108 gram.
66	2.99	30	+	11					
67	3.06	30	—	12					Weight: 5050 grams.
78	2.83	31	3.7	11	68 8	24 89			
79	2.43	28	3.6	11	66 8	26 89			{ Hypoxanthine injection: 0.121 gram.
80	1.38	34	17.8	12					
81	1.98	24	4.0	10					{ Guanine injection: 0.153 gram.
82	1.44	31	—	—					
83	1.58	25	2.5	10	67 7	27 91			
84	2.67	30	3.4	11	68 8	25 90			
85	2.23	58	5.8	21					
86	2.59	30	3.2	12	66 7	27 90			
87	2.42	29	2.8	11	68 7	26 91			
88	2.44	29	3.6	13	64 8	28 89			
89	1.90	57	6.0	26					
90	3.03	41	3.8	18					
91	2.36	30	3.1	11	68 7	25 91			

TABLE II—Continued.

PERIOD	OUTPUT OF 48 HOURS				PERCENT- AGE OF TOTAL PURINE- ALLANTOIN N AS			URICOLYTIC INDEX	REMARKS
	Total N	Allantoin N	Uric Acid N	Purine Base N	Allantoin	Uric Acid	Purine Bases		
	grams	mgms.	mgms.	mgms.					
92	2.02	29	3.6	10	68	8	23	89	
93	2.04	27	3.2	10	67	8	25	89	
96	2.67	28	3.4	12	65	8	28	89	Weight: 5100 grams.
97	2.63	40	5.8	33				87	{ Adenine injection: 0.124 gram.
98	2.84	31	3.2	12				91	
99	2.77	28	3.7	12	64	8	27	88	
100	2.86	69	6.4	27				92	{ Xanthine injection: 0.152 gram.
101	3.09	34	3.4	14				91	
102	2.96	29	3.1	12	66	7	27	90	Weight: 4950 grams.
105	2.68	29	3.5	11	67	8	25	89	Weight: 4950 grams.
106	2.78	29	3.1	11	67	7	26	90	
107	2.91	64	6.2	36				91	{ Sodium nucleate injection: 1.036 grams.
108	1.82	32	3.0	12				91	
109	2.60	29	2.6	10	70	6	24	92	
110	2.89	36	24.0	11					{ Third uric acid injection: 0.147 gram.
111	2.78	32	3.9	12					
112	2.79	27	4.8	11					Weight: 4850 grams.
113	2.87	28	3.6	—				89	

reason it is usually impossible to base any useful inferences upon the total excretion of nitrogen. The food consumed was in any case sufficient to prevent any serious loss of weight.

All analyses were made, as formerly, upon the combined urine of forty-eight hours.

Details not recorded in the foregoing table will be found in the following abstract prepared from the daily protocols. It is to be understood that each operation was performed on the first day of its respective period.

*First uric acid injection:* period 46. 0.200 gram uric acid (67 mgms. N) dissolved in dilute NaOH solution injected from a burette into the loose subcutaneous tissue of the left flank. No anesthetic. Serious local and general effects ensued. The monkey ate hardly anything for a week afterwards, and was obviously very ill. The tissues round the point of injection necrosed, the skin sloughed, and an ugly ulcer developed. After eight days the animal had to be removed from its cage for treatment.

*First uric acid feeding:* period 61. 0.103 gram uric acid (34 mgms. N) in dilute sodium hydroxide solution was added to the milk ration. Ingestion was quantitative. The general condition of the animal was not perceptibly affected.

*Second uric acid feeding:* period 65. 0.198 gram uric acid (66 mgms. N) administered in the same way, without obvious outward effect.

*Second uric acid injection:* period 80. Under anesthesia 12.1 cc. of saturated lithium carbonate solution containing 0.108 gram uric acid (36 mgms. N) were injected subcutaneously from a burette. Most of the milk of that day's ration was refused, and for two days the animal was rather inactive. Otherwise this injection did not affect her health.

*Hypoxanthine injection:* period 85. Monkey anesthetized, and 21.0 cc. of 0.4 per cent lithium carbonate containing 0.121 gram hypoxanthine (50 mgms. N) subcutaneously injected from a burette. The injection had no effect other than a diminution of appetite during the remainder of the day.

*Guanine injection:* period 89. Under anesthesia injected from a burette into the left side of the back 49.5, and into the right, 72.1 cc. of 0.4 per cent lithium carbonate containing in all 0.153 gram of guanine (71 mgms. N). During the remainder of the day the monkey ate practically nothing, and vomited several times. The urine was not contaminated. Next day the appetite and activity of the animal were subnormal, but there was no more vomiting. During the third and fourth days (period 90) the right side was found to be slightly swollen and very tender; activity greater, appetite still poor. In period 91 the animal returned to practically normal condition.

*Adenine injection:* period 97. Anesthesia, and injection from a burette of 0.124 gram adenine (64 mgms. N) in 0.4 per cent lithium carbonate. No local or general disturbance.

*Xanthine injection:* period 100. Monkey anesthetized, and 0.152 gram xanthine (56 mgms. N) in 0.4 per cent lithium carbonate injected from a burette. Absolutely no effect perceptible.

*Sodium nucleate injection:* period 105. From a carefully calibrated hypodermic syringe there were injected at 10 a.m. 1.5 cc., at noon 3 cc., at 4 p.m. 4 cc. and at 7.30 p.m. 5 cc. of a solution of sodium nucleate in sterile salt solution. The total amount of nucleate thus introduced was 1.036 grams (90 mgms. purine N). No anesthetic was employed. The injections produced no local or general disturbance.

*Third uric acid injection:* period 110. At 9.30 a.m., 12.30, 4 and 7.30 p.m., respectively, injected without anesthetic 3.6, 4.0, 4.0 and 4.4 cc. (total 16.0 cc.) of a 0.4 per cent lithium carbonate solution containing altogether 0.147 gram uric acid (49 mgms. N). No after effect of any kind was noticed.

## THE OUTPUT OF ENDOGENOUS PURINES.

In so far as the colorimetric method has enabled us to measure the *uric acid* output of our subject, we are in a position to supplement and correct our earlier report upon the endogenous purine excretion. In the later sections of Table II we have, for each period not obviously abnormal, calculated upon the fresh basis now available the distribution of nitrogen among bases, uric acid and allantoin. The figures show that the uric acid fraction, accounting constantly for 7 or 8 per cent of the whole, is not precisely insignificant. It is decidedly more prominent in the monkey than, for instance, in the dog. The predominance of bases over uric acid remains nevertheless sufficiently striking, the former being three to four times as abundant as the latter. With the introduction of uric acid into the calculation, the allantoin ratio, in the sense defined in our former paper, becomes even lower than before. Its average value is only 67.

If we assume that excretion proceeds neither more nor less rapidly than formation, the magnitude of the allantoin ratio will depend upon the relative velocities of all the reactions involved in the catabolism of purines. The data allow us to estimate the rate of none but the last of these, the one commonly, if not very happily, described as "uricolysis." A numerical expression can be obtained for it by calculating the allantoin nitrogen as a percentage of the sum, allantoin N + uric acid N.<sup>5</sup> This expression we propose to call for convenience the "uricolytic index." Under that name it has been entered in Table II for all periods in which it has not been modified by the *direct* introduction of uric acid. It will be seen that it fluctuates with very slight variations about the value 90. This would indicate in the monkey a capacity for uricolysis which is certainly high, but which is distinctly inferior to that generally possessed by the dog. It is at least possible that we have here the first sign of that loss of uricolytic power which is so nearly complete in the higher apes and man.

<sup>5</sup> Wiechowski prefers to calculate the per cent of "uric acid decomposed," the number representing which is essentially the same as our "uricolytic index." See *Biochem. Zeitschr.*, xix, p. 368, 1909.

## THE FATE OF EXOGENOUS PURINES.

*a. After oral administration.*

In two of the experiments a small quantity of uric acid was fed by the mouth. In the first (period 61) the uric acid, containing 34 mgms. nitrogen, simply disappeared, excepting in so far as a barely appreciable fraction passed without change into the urine. In the second (period 65) the result was essentially the same; the uric acid fed contained 66 mgms. of nitrogen, of which 5, or 7.6 per cent, was recovered unaltered, while the remainder could not be accounted for in the urine at all. In both cases we succeeded in isolating from the feces a few crystals which had the appearance of uric acid. Their identification was not complete, but even if it had been, the amount involved was utterly insignificant. We believe it probable that a large part of the uric acid was in each instance destroyed by the action of intestinal bacteria. The following experiment lends support to this explanation.

*Experiment.* A little more than a gram of uric acid was dissolved in 100 cc. of 0.4 per cent lithium carbonate. This solution was diluted to 250 cc., and sterilized by boiling. Two 25-cc. portions were then removed. One (A) was stirred up with about 5 grams of fresh monkey feces, and further diluted with 25 cc. of water. To the other (B), which served as a control, 25 cc. of water alone were added. Both were kept at 40°C. for three days. (Even at this temperature some urate crystallized from B.) From each, after dilution, the uric acid was then precipitated with copper, the precipitate in the case of A proving to be very minute. There was finally isolated from B 114 mgms. of uric acid, so that here very little must have undergone decomposition. From A none could be isolated, and according to a colorimetric determination the amount present could not have exceeded 2 mgms. At least 98 per cent had therefore been destroyed by bacteria.

Bacteria which destroy uric acid have been described by Ulpiani<sup>6</sup> and by Liebert,<sup>7</sup> and evidently organisms of like capacity inhabit the alimentary tract of the monkey. It is quite likely then that much of the uric acid fed never entered the blood stream.

<sup>6</sup> Ulpiani: *Atti della R. Accad. dei Lincei*, xii, p. 236; cited from Wiechowski: *Beitr. z. chem. Physiol.*, ix, p. 295, 1906-7.

<sup>7</sup> Liebert: *K. Akad. Wetensch. Amsterdam, Versl. Wis. en Naturk. Afdel.*, xvii, p. 990, 1908-9; cited from *Exp. Sta. Record*, xxiv, p. 530.

Some, however, did, for some appeared as such in the urine. It is therefore surprising that to all appearance none was converted into allantoin. This puzzling circumstance we are unable fully to explain. We shall return to it in discussing the uric acid injections, where a similar deficit of allantoin has to be accounted for.

*b. After subcutaneous injection.*

The significant results of the injection experiments are exhibited in Table III. From this table we have decided to exclude the first experiment with uric acid (period 46). It is one which resulted in the elimination during eight days of such an excess of allantoin and uric acid as would account for precisely 100 per cent of the material introduced. But an examination of the protocols shows that during most of that time the animal had an extensive area of necrosis, and we are of opinion that this of itself was enough to raise the purine metabolism to a higher level. This opinion is strengthened by the facts, that the output of purine bases rose above the normal, and that the excretion of uric acid had apparently not yet come to an end when the experiment was terminated. Under these circumstances the result is really valueless. If it had not already appeared in a preliminary communication,<sup>8</sup> published before we had made other experiments

TABLE III.

PERIOD	SUBSTANCE	PURINE N ADMINIS- TERED IN MILLIGRAMS	RECOVERED				PER CENT OF SUB- STANCE EXCRETED UNCHANGED	
			Milligrams N					Per cent of theory
			Allan- toin	Uric Acid	Bases	Total		
80	Uric acid	36	8	14		22	61	40
110	Uric acid	49	12	23		35	71	47
89	Guanine	71	38	2	20	60	99-105	-28
100	Xanthine	56	35	2	17	54	97	30
97	Adenine	64	15	3	21	39	69-76	-33
85	Hypoxanthine	50	28	3	9	40	80	-18
107	Sodium nucleate	90	38	3	26	69	88-96	2

<sup>8</sup> Hunter and Givens: *Orig. Comm. Eighth Internat. Congress Chem.*, xix, p. 149, 1912.

upon the point, it would have been omitted altogether from the present paper.

By way of elucidation of this table it should be mentioned that in calculating the excess excretion of any experimental period we have as formerly taken for our base the average between the period immediately preceding the injection and the next certainly normal one following; that since with the small quantities involved an error of 1 mgm. would make an appreciable difference in the "percentage recovery," the figures under the latter head form a rough approximation only; that where guanine, adenine, and sodium nucleate are concerned, account has been taken of the fact that the uric acid and allantoin produced from them contain but four-fifths of the original amino-purine nitrogen; and that where two alternatives are offered for the percentage recovered, the lower rests on the assumption that the extra bases are amino-purines, while the higher takes them as oxy-purines, and the truth lies probably between the two.

The injection of sodium nucleate involves yet another consideration. Here there was the possibility that some nucleic acid might be excreted unchanged, or at least before its bases had been liberated. Accordingly we made in periods 106, 107 and 108 determinations not only of the free bases, but also of the total bases precipitable by copper after hydrolysis of the urine. The result showed that the urine of the monkey normally contains a small quantity (about 2 mgms.) of purine nitrogen in combined form, and that this was increased by 2 mgms. on injection of sodium nucleate. That substance escaped unchanged, therefore, to the extent of only about 2 per cent, an observation which has been entered in the above table, and which has been duly taken account of in computing the proportion recovered.

*Fate of guanine.* The literature contains a few experiments upon the subcutaneous or intravenous injection of guanine, but in none of them were determinations made of the allantoin which forms the chief product of its metabolism. In our experiment the guanine introduced was quantitatively accounted for. The recovery indeed is almost *too* complete, and, when considered in connection with the rather severe effect produced upon the animal's health, leads one to doubt somewhat the validity of the result. Nevertheless, on a fair review of all the circumstances, especially in relation to the quite unexceptionable result with xanthine, we feel justified in claiming the experiment as conclusive. The metabolism of guanine proceeds to the production of allantoin, and allantoin is the end of the process.

Some of the guanine without doubt was excreted unchanged; just how much it is impossible to say, since the extra basic nitro-

gen may have been either guanine or xanthine. Schittenhelm and Bendix<sup>9</sup> could isolate no guanine from the urine of rabbits after subcutaneous and intravenous injections of the same, while xanthine and uric acid were identified. Mendel and Lyman<sup>10</sup> also obtained xanthine in similar circumstances. We have no evidence as to which base in our case was being eliminated. As guanase is abundantly present in the tissues of the monkey,<sup>11</sup> it is probable that xanthine predominated; in which case the proportion of unaltered guanine excreted must have been considerably less than 28 per cent.

According to the accepted view of the matter, all the guanine converted into allantoin must have passed through the intermediate stage of uric acid. An increase in the output of the latter substance was therefore to be expected, and did actually occur. It was, however, exceedingly small, corresponding to but 3.5 per cent of the guanine introduced. We take occasion here to point out that every injection of a purine base, free or in combination, was followed by a rise in uric acid of the same small order of magnitude. Moreover, excepting with adenine, the relation of the exogenous uric acid to the exogenous allantoin was approximately the same as that normally existing between their endogenous values. Accordingly, as appears in Table II, the administration of any purine except uric acid itself has no appreciable effect upon the uricolytic index. All the data indeed agree in indicating that uric acid, so far as it arises in intermediary metabolism, and is not merely introduced in unusual quantities from without, is to the extent of 90 per cent converted into allantoin; and that exogenous purines, once they have effectively entered the metabolic stream, undergo precisely the same fate as those set free within the organism.

*Fate of xanthine.* The fate of xanthine after parenteral introduction has been studied only by Lewinthal.<sup>12</sup> Besides performing an experiment upon himself, that observer injected an impure xanthine preparation into the ear vein of a rabbit. He recovered

<sup>9</sup> Schittenhelm and Bendix: *Zeitschr. f. physiol. Chem.*, xliii, p. 365, 1905.

<sup>10</sup> Mendel and Lyman: this *Journal*, viii, p. 115, 1910.

<sup>11</sup> See Wells: this *Journal*, vii, p. 171, 1910.

<sup>12</sup> Lewinthal: *Zeitschr. f. physiol. Chem.*, lxxvii, p. 259, 19 ~

33 per cent unchanged, and 40 per cent as uric acid; but, since allantoin was not determined, the result is from a quantitative point of view of very limited interest.

Our own experiment with this substance was the most convincing of the series. The injection was followed by absolutely no symptoms whatever, the only effect upon total nitrogen output was a slight and perhaps accidental rise in the period following, and the recovery of the purine was quantitative. A large proportion, 30 per cent, is excreted unchanged. In fact there escapes catabolism more of the xanthine, than of any other injected purine except uric acid and, possibly, adenine. The explanation for the first two is probably the same, namely, that in the monkey both uricase and xanthine-oxidase are confined to one organ, the liver,<sup>13</sup> and that the action of this may be partly escaped by material parenterally introduced. The xanthine not excreted as such is entirely accounted for as uric acid and allantoin.

*Fate of adenine.* While injections of adenine have been several times recorded, none have been accompanied by accurate allantoin determinations in the urine. The only ones in which any of the products were quantitatively estimated are those of Mendel and Lyman.<sup>14</sup> They found that both with rabbits and dogs a relatively large porportion of adenine was eliminated unchanged, while the increase in uric acid was very small. It is noteworthy that adenine is not an altogether innocuous substance, and has been observed to produce in dog, rat and rabbit a nephritic condition accompanied by the formation and deposition in the kidney of 6-amino-2,8-oxypurine.<sup>15</sup>

Our experiment with the monkey was followed by no untoward symptoms of any kind; appetite was unimpaired, albuminuria was absent, and the nitrogen output did not abandon the normal level. Nevertheless the purine balance was less satisfactory than with any other base. Only about 70 per cent of the dose was recovered in identified combinations. Of this about half was found in the base fraction, which, to judge from the experience

<sup>13</sup> Wells: *loc. cit.*

<sup>14</sup> Mendel and Lyman: *loc. cit.*

<sup>15</sup> Minkowski: *Arch. f. exp. Path. u. Pharm.*, xli, p. 375, 1898; Nicolaier: *Zeitschr. f. klin. Med.*, xlv, p. 359, 1902; Ebstein and Bendix: *Virchow's Archiv*, clxxviii, p. 464, 1904.

of Mendel and Lyman, was probably chiefly composed of unchanged adenine. Assuming the case to be so, one-third of the substance escaped disintegration. This would argue for our monkey a rather deficient equipment of adenase. Wells,<sup>16</sup> it is true, found this enzyme widely distributed in the tissues of *Macacus rhesus*; but of another monkey—*Cebus apella* (?)—he reports that the liver was able to deaminate only 50 per cent of added adenine within eleven days. It is of interest in this connection that in the human organism adenase, according to Miller and Jones,<sup>17</sup> is entirely lacking.

The increase of uric acid produced by adenine was much the same as with the other bases. The large deficit falls therefore to all appearance mainly upon the allantoin fraction. Beyond the known toxicity of adenine we have at present no clue which would assist in its explanation. Before indulging in hypotheses it would be necessary to have the observation duplicated. Certainly we cannot from one negative result conclude that adenine has a metabolic history quite dissimilar from that of guanine.

*Fate of hypoxanthine.* The effect of hypoxanthine injections upon the output of purines has been studied by Burian and Schur<sup>18</sup> and by Mendel and Lyman,<sup>19</sup> but in none of their experiments was allantoin determined. By including the latter in the reckoning we accounted for 80 per cent of the hypoxanthine which we introduced. This value is neither so high that we can claim a quantitative recovery, nor so low that the deficiency might not be due to errors of analysis. It may not be a mere coincidence that among the bases it is precisely the closely related pair, adenine and hypoxanthine, that yields us the lowest figures; on the other hand it is difficult to believe that hypoxanthine gives any product of metabolism that is not met with in the case of xanthine.

Very little hypoxanthine was excreted unchanged. It is worth recording, that during the evaporation of the purine solution obtained from the urine of period 85 there separated a considerable amount of semi-crystalline material, which was not uric acid, could hardly have been hypoxanthine, and was therefore almost

<sup>16</sup> Wells: *loc. cit.*

<sup>17</sup> Miller and Jones: *Zeitschr. f. physiol. Chem.*, lxi, p. 395, 1909.

<sup>18</sup> Burian and Schur: *Arch. f. d. ges. Physiol.*, lxxxvii, p. 239, 1902.

<sup>19</sup> Mendel and Lyman: *loc. cit.*

certainly xanthine. The hypoxanthine which passed unaltered into the urine must in that case have been less than 18 per cent of the quantity administered, a smaller proportion than with any other purine. It is a confirmation of this conclusion, that *all* the products of exogenous metabolism in this particular experiment bear to one another precisely those ratios that are characteristic of the endogenous metabolism. In Mendel and Lymans' hypoxanthine experiment with the rabbit the result was very different, nearly half of the substance being excreted unchanged.

*Fate of sodium nucleate.* Practically none of the injected nucleic acid reappeared in the urine unaltered. The distribution of the dose given over a period of nine and a half hours may have contributed somewhat to its complete disintegration. Within the limits of our analytical methods the liberated purines are quantitatively accounted for. While the preponderance of bases among the products is greater than might have been expected, the general inference to be drawn from the result is clear enough: exogenous and endogenous purines undergo identical metabolic changes, and the sum of the urinary purines and allantoin represents the total catabolism of purines in the organism. The results with guanine, xanthine, and to a certain extent hypoxanthine, afford ample confirmation of this thesis, which cannot be held to be disproved by the single failure with adenine.

*Fate of uric acid.* We have left this to the last because the experiments with uric acid apparently invalidate the conclusion just enunciated. Perfectly consistent with one another, they are in two respects at variance with our other results. The high uricolytic index of our animal would certainly lead one to expect that it would readily dispose of injected uric acid. On the contrary we find that 40 to 50 per cent is excreted unchanged. This is more than in any other similar experiment upon lower mammals reported in the literature, the nearest figure being 38 per cent after intravenous injection of 0.167 gram of uric acid into a rabbit.<sup>20</sup> Again, of the uric acid not simply excreted, only a small fraction appears as extra allantoin, so that 30 to 40 per cent remains quite unaccounted for. Both difficulties would be explicable if we assumed, (1) that allantoin is formed for the most part directly from purine bases without passing through the

<sup>20</sup> Mendel and Lyman: *loc. cit.*

stage of uric acid,<sup>21</sup> (2) that the production of uric acid within the organism is therefore normally very small, and the power to deal with it possibly much more limited than we have supposed, and (3) that such uricolysis as occurs may lead to other products than allantoin. Such a scheme would explain also the feeding experiments with uric acid, and would be consistent with the high base and low uric acid content of the urine. But there is no direct evidence in favor of it, and it certainly bears in all its aspects the appearance of improbability. It is much more likely that the high output of unaltered uric acid is related to the fact, already mentioned, that the monkey's only uricolytic organ is the liver, and that the deficit depends on such factors as temporary retention or local deposition with subsequent gradual elimination. Support for such a conception is actually to be found in the experimental data. Thus the uric acid of period 81, though not so high that we can be certain of its being in part exogenous, is higher than that of any other normal period; the same is to be said of the allantoin output of period 82, which moreover is decidedly above that of the periods on either side of it. Similarly after the third uric acid injection there is distinct evidence of a belated elimination of uric acid in period 112.

Still another circumstance deserves to be noted. In our earlier paper it was shown that the injection of allantoin, or even sometimes the feeding of sodium nucleate, may depress temporarily the normal level of the allantoin output. It appears now that the same phenomenon may be brought about by the presence in the circulation of uric acid; witness the figures for periods 81 and 83. If this be so, it is conceivable that the actual exogenous excretion of allantoin may be partly concealed by a simultaneous drop in the endogenous quota, and that we are dealing therefore with a deficit more apparent than real.

If to these considerations be added the possible toxic effect of uric acid, it will, we believe, be apparent, that the results with uric acid are not altogether irreconcilable with the conclusions drawn from the other experiments.

<sup>21</sup> Wiechowski (in Ellinger's *Analyse des Harns*, 1913, p. 929) supposes that such a state of affairs may perhaps account for all those cases where bases are excreted in excess of uric acid.



## THE NITROGEN EXCRETION OF THE MONKEY.

By ANDREW HUNTER AND MAURICE H. GIVENS.

(From the Department of Physiology and Biochemistry, Cornell University Medical College, Ithaca, N. Y.)

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Systematic urine analyses including in their scope more than one or two special constituents have been carried out for a surprisingly limited number of species, even among the mammalia. From the point of view of comparative biochemistry this is to be regretted, and any information ought to be welcome, which helps to fill the gap here existing in our knowledge. We have accordingly decided to put on record a few precise data relating to the urine of a monkey, *Cercopithecus callitrichus*, living upon a standard diet in a condition of at least approximate nitrogen equilibrium. These data were collected incidentally to a special investigation already reported in this *Journal*,<sup>1</sup> and include all the more important nitrogenous excreta.

The monkey was a female of which the weight during the first series of observations here recorded was 4.7, during the second, 4.9 kgms. The daily diet of the first series consisted of 200 cc. of whole milk, 200 grams of bananas, and 20 grams of peanuts (calculated to contain 2.09 grams of nitrogen and 418 calories); for the second, this was reduced to 150 cc. of milk, 150 grams of bananas and 25 grams of peanuts (1.91 grams of nitrogen and 363 calories). On each day included in the tables below the consumption of the allowance was quantitative. The urine was collected in forty-eight-hour periods. In its analysis we employed the method of Kjeldahl for *total nitrogen*; of Benedict for *urea*; of Folin for *ammonia*, *creatine*, and *creatinine*; of Wiechowski for *allantoin*; and of Krüger-Schmid (omitting the use of manganese dioxide) for *purines*. In connection with the last determination *uric acid* was

<sup>1</sup> Hunter and Givens: this *Journal*, xiii, p. 371, 1912; and xvii, p. 37, 1914.

TABLE I.  
Series I.

PERIOD	ESTIMATED NITROGEN INCOME	GRAMS NITROGEN IN FORTY-EGHt HOURS							PERCENTAGE DISTRIBUTION					FECAL NITROGEN	
		Total	Urea	Ammonia	Creatinine	Allantoin	Purines	Undetermined	Urea	Ammonia	Creatinine	Allantoin	Purines		Undetermined
	grams														grams
1	4.18	3.58	3.14	0.060	0.136				87.7	1.67	3.8				2.75
2	4.18	3.71	3.23	0.065	0.132				87.1	1.75	3.6				
3	4.18	3.62	3.17	0.049	0.126				87.6	1.35	3.5				
4	4.18	3.67	3.20	0.053	0.121	0.030	0.010	0.256	87.2	1.44	3.3	0.82	0.27	7.0	
5	4.18	3.59	3.09	0.054	0.134	0.029	0.009	0.274	86.1	1.50	3.7	0.81	0.25	7.6	
6	4.18	3.81	3.28	0.066	0.132	0.031	0.009	0.292	86.1	1.73	3.5	0.81	0.24	7.6	
7	4.18	3.64	3.11	0.038	0.128	0.031			85.5	1.04	3.5	0.85			
Daily average.....		1.83	1.59	0.028	0.065	0.015	0.0047	0.137	86.8	1.53	3.5	0.82	0.26	7.5	0.20

TABLE II.  
Series II.

PERIOD	ESTIMATED NITRO-GEN INCOME	GRAMS NITROGEN IN FORTY-EIGHT HOURS								PERCENTAGE DISTRIBUTION						
		Total	Urea	Ammonia	Creatinine	Allantoin	Purines	Hippuric Acid	Undetermined	Urea	Ammonia	Creatinine	Allantoin	Purines	Hippuric Acid	Undetermined
16	3.82	3.50	3.05	0.027	0.135	0.030	0.010	0.046	0.202	87.1	0.77	3.9	0.86	0.29	1.34	5.8
17	3.82	3.60	3.08	0.044	0.140	0.027	0.011	0.046	0.232	85.6	1.22	3.9	0.75	0.31	1.28	7.0
18	3.82	3.47	3.01	0.048	0.145	0.030	0.011	0.046	0.180	86.8	1.38	4.2	0.86	0.32	1.32	5.2
19	3.82	3.64	3.05	0.072	0.137	0.030	0.011	0.046	0.294	83.8	1.98	3.8	0.82	0.30	1.26	8.1
20	3.82	3.74	3.26	0.037	0.140	0.031	0.011	0.046	0.215	87.2	0.99	3.7	0.83	0.29	1.23	5.8
Daily average.....	1.91	1.80	1.55	0.023	0.070	0.015	0.0054	0.023	0.114	86.1	1.28	3.9	0.83	0.30	1.28	6.3

TABLE III.  
*Average daily excretion.*

	GRAMS SUBSTANCE			MGMS. N PER KG. BODY WEIGHT
	Series I	Series II	Final averages	
Total nitrogen.....	1.83	1.80	1.815	378.0
Urea.....	3.41	3.32	3.37	327.0
Ammonia.....	0.034	0.028	0.031	5.3
Creatinine.....	0.175	0.188	0.182	14.1
Allantoin.....	0.042	0.042	0.042	3.1
Uric acid.....			0.0049	0.34
Purine bases (as xanthine).....	0.013	0.015	0.014	1.1
Hippuric acid.....		0.29	0.29	4.8

always sought, but could never be isolated. The value for uric acid given in the last two columns of Table III is interpolated from a later series of analyses in which that substance was estimated by an application of the Folin-Macallum colorimetric method.<sup>2</sup> The quantity of urine available did not permit serial determinations of any other constituents, but in connection with the second set of observations we estimated, by the method of Folin and Flanders,<sup>3</sup> the *hippuric acid* content of a composite sample made up of equal aliquot parts of each period's urine. The nitrogen content of the *feces* was determined only for the first series.

The results are collected in Tables I, II and III, which explain themselves.

In series I (Table I) the average daily loss of nitrogen was  $1.83 + 0.20 = 2.03$ . The estimated income corresponding was 2.09. For the time then the animal must have been very nearly in nitrogen equilibrium; and the same is probably true for series II.

In other communications<sup>4</sup> we have sufficiently insisted upon the characteristics of *purine* metabolism in the monkey. Here

<sup>2</sup> For the manner in which we applied this method to the monkey urine, see our paper in the present number of this *Journal*. In the purine determinations, as practiced for the early analyses here reported, the uric acid was probably all but entirely destroyed. At any rate it was later found that the purine bases alone accounted for as much nitrogen as is here assigned to total purines.

<sup>3</sup> Folin and Flanders: this *Journal*, xi, p. 257, 1912.

<sup>4</sup> Hunter and Givens: *loc. cit.*

it will only be pointed out that the nitrogen derived from the breakdown of purines constitutes a little over 1 per cent of the total output, in which respect the monkey shows an apparent similarity to man; but on the basis of body weight the purine-allantoin nitrogen eliminated (4.5 mgms. per kilogram per day) is about twice as great as in the human subject.

As for the metabolism of *proteins* the data of the tables show that in the monkey it exhibits hardly any peculiarities that call for special comment. The total nitrogen output in relation to body weight is one and a half to two times as great as is usual in man; but is smaller than it would be likely to be in appropriately fed dogs of equal size, or than it has been found in rats living upon an unrestricted diet of cracker meal and water.<sup>5</sup> The urea and creatinine bear such relations to the total nitrogen as might be met with in any known mammalian urine. The ammonia ratio is rather low, as would be expected on a vegetable diet. The *creatinine coefficient* of our monkey is in the neighborhood of 14, which is higher than that of man. It may be stated that, while we possess a very much longer series of creatinine determinations than is here reproduced, the tables given happen to include the lowest as well as the highest values ever observed. The examination of the urine for creatine yielded results which were either entirely negative, or could hardly be said to lie beyond the experimental error of the determination.

<sup>5</sup> See Folin and Morris: this *Journal*, xiv, p. 509, 1913.



# ON THE RELATIVE INTOLERANCE OF THE SHEEP TO SUBCUTANEOUS ADMINISTRATION OF GLUCOSE.

By ANDREW HUNTER AND REUBEN L. HILL.

(From the Department of Physiology and Biochemistry, Cornell University, Medical College, Ithaca, N. Y.)

(Received for publication, December 31, 1913.)

In 1902 it was shown by Scott<sup>1</sup> that dogs may tolerate without a trace of glycosuria the subcutaneous injection of as much as 5 grams of glucose per kilogram of body weight. This observation has been confirmed by Underhill and Closson<sup>2</sup> who found, moreover, that the power to utilize subcutaneously introduced glucose is as well developed in the rabbit as in the dog. It does not by any means follow that every animal is endowed with the same capacity. On the contrary there are indications that the assimilation limit for carbohydrates may vary widely from species to species. Reference may be made particularly to a recent paper by Carlson and Drennan<sup>3</sup> from which it appears that the pig "has a lower tolerance for dextrose, bread or cooked starch given by the mouth than any other species so far studied;" and that, for instance, "a normal pig weighing 8-10 kilos shows marked alimentary glycosuria when given 2.5 grams of dextrose." While the pig was not made the subject of any *injection* experiments, it is exceedingly unlikely that an animal thus behaving would be capable of assimilating any considerable amount of glucose by the subcutaneous route.

Some time ago one of us observed that a thyro-parathyroid-ectomized lamb, upon the subcutaneous administration of 5 grams per kilogram of glucose, excreted unaltered as much as 50 per cent of the dose.<sup>4</sup> In view of the results of Scott and of Under-

<sup>1</sup> Scott: *Journ. of Physiol.*, xxviii, p. 107, 1902.

<sup>2</sup> Underhill and Closson: *this Journal*, ii, p. 117, 1906.

<sup>3</sup> Carlson and Drennan: *ibid.*, xiii, p. 465, 1912-13.

<sup>4</sup> The details of this observation will be published shortly.

hill and Closson upon dogs and rabbits, this was at first taken to indicate that the loss of the glands had produced a decided diminution of carbohydrate tolerance. When this conclusion was tested by ascertaining the reaction of normal sheep to the same procedure, it was found to have been rather prematurely drawn. The operated animal in fact turned out to have actually a smaller tendency to glycosuria after the parenteral introduction of sugar, than had the normal sheep. The results with the latter are so surprising, and, in connection with Carlson and Drennan's on the pig, so interesting, that we have thought them worthy of a brief communication.

We made six subcutaneous injections of glucose upon three healthy sheep.<sup>5</sup> In every case the glucose was dissolved in physiological saline so as to make an approximately 20 per cent solution; the amount to be introduced was calculated from the result of a Benedict-Fehling titration after the solution had been sterilized by boiling. The injection was made in one dose, excepting in experiments 1 and 2, where it was divided into two. The sugar excreted was determined by the Benedict-Fehling method,<sup>6</sup> controlled in several cases by the polarimeter. The animals were allowed to eat as they pleased and the urine was collected daily without catheterization. Consequently the nitrogen figures permit no deduction as to the possible effect of the injections upon protein metabolism. The urine was regularly tested for albumin and acetone, but on no occasion was either detected.

The quantitative details and results of the experiments may be ascertained from the accompanying table. After every injection some sugar escaped through the kidneys. The excretion began very early; in experiment 2 a specimen of urine caught fifteen minutes after the operation contained a trace of sugar, and in experiment 3 the urine had acquired strong reducing properties within an hour. Only on one occasion was the elimination continued into the next day. It will be seen that the percentage of sugar recovered does not bear any strict relation to the relative size of the dose. The general conclusion to be drawn is none the

<sup>5</sup> For placing at our disposal the sheep employed in the experiments we are indebted to the courtesy of Prof. H. H. Wing of the Department of Animal Husbandry in this University.

<sup>6</sup> Benedict: this *Journal*, iii, p. 101, 1907.

less obvious. The sheep's capacity to deal with subcutaneously administered glucose is greatly inferior to that of the dog. While the latter can dispose of 5, or even 7, grams per kilogram, the former has difficulty in retaining as small a proportion as 0.5 gram. How much lower the absolute limit lies we did not think it worth while to ascertain; nor have we tested the degree to which the sheep is liable to *alimentary glycosuria*.

It is rather surprising to find that an animal of which the diet naturally contains a large proportion of carbohydrates should be less tolerant of sugar than the flesh-eating dog. Diet habits indeed appear to have little to do with the matter; for the rabbit, as Underhill and Closson found, reacts like the carnivora, while the sheep allies itself with the omnivorous pig. The factors actually concerned would make an interesting subject of inquiry.

SHEEP	WEIGHT	EXPERIMENT NUMBER	GLUCOSE INJECTED		EXCRETION		RECOVERY	DIET
			Grams	Grams per kilo- gram	N	Glucose		
	<i>kgms.</i>				<i>grams</i>	<i>grams</i>	<i>per cent</i>	
A	56.9	1	146	2.6	6.83	0	13.1	Corn and clover
					18.46	0		
					7.79	16.7		
					9.95	2.5		
					6.20	0		
B	39.0	2	104	2.7	6.42	0	9.5	Corn and clover
					7.31	9.9		
					13.20	0		
		3	58	1.5	9.17	0	22.2	
					7.91	0		
		4	38.2	0.98	5.78	12.9	16.5	
					7.74	0		
5.74	6.3							
C	28.7	5	14.3	0.5	6.90	0	8.4	Oats and alfalfa
					6.27	1.2		
		6	28.7	1.0	9.95	6.1	21.2	
					?	0		



## A NOTE ON THE DETERMINATION OF CREATININE AND CREATINE IN MUSCLE.

By VICTOR C. MYERS AND MORRIS S. FINE.

(From the Laboratory of Pathological Chemistry, New York Post-Graduate  
Medical School and Hospital.)

(Received for publication, December 31, 1913.)

The value of the analysis of body tissues and fluids in the study of problems in metabolism has been amply demonstrated during the past two years. New methods for the amino nitrogen, non-protein nitrogen, urea and uric acid of the blood and tissues have greatly modified our views regarding nitrogenous metabolism.<sup>1</sup> The relation of the creatine content of the muscle tissue to creatine-creatinine metabolism has been considered in contributions from this laboratory.<sup>2</sup> The data have, we believe, given us suggestive information on this subject. On this account it has been thought desirable to describe the methods which have been found most satisfactory.

In an earlier paper<sup>3</sup> we briefly described the methods which had been employed in determining creatine (total creatinine) in muscle tissue. Since that time we have had occasion to determine any preformed creatinine that might be present as well as the creatine. While our original method is quite suitable for the determination of creatine (total creatinine) alone, it was found essential to introduce certain modifications in order that both creatine and creatinine might be determined in the same sample. In the original method the proteins were removed by coagulation, but since it is necessary to avoid heat in the instances where creatinine is to be determined, the proteins are removed in this latter case with the aid of colloidal precipitants. Colloidal iron, freshly pre-

<sup>1</sup> We refer to recent publications of Folin and Denis, and Van Slyke in this *Journal*.

<sup>2</sup> Myers and Fine: *ibid.*, xiv, p. 9; xv, pp. 283 and 305; xvi, p. 169, 1913.

<sup>3</sup> *Ibid.*, xiv, p. 12, 1913.

cipitated and washed ferric hydroxide and alumina cream have been used for this purpose, our experience having been most extensive with the last named precipitant, which we have found very suitable for this purpose. Since we began the use of the present method,<sup>4</sup> a paper has appeared by Beker<sup>5</sup> in which he has employed colloidal iron to excellent advantage in the determination of creatine (total creatinine) in various body tissues.

In interpreting the percentage figures for the creatine content of the muscle it is very important to ascertain the moisture content of the muscle, while the nitrogen content is a further valuable check upon this data.

#### METHODS.

The muscle tissue, carefully freed from connective tissue and fat, is ground in a small meat chopper and samples *at once* taken for the creatine (and creatinine) determination, also for the moisture and nitrogen if these determinations are to be made. The sample intended for the creatine (and creatinine) estimation is placed in a 200-cc., large-neck, glass stoppered bottle and 95 per cent alcohol slowly added until the tissue is completely covered, stirring constantly to break the muscle up very finely. The tissue is preserved in this way at 0°C. until the extraction is made. For comparative work it is best to employ a uniform set of dilutions upon the extract, the quantity of muscle tissue depending upon the percentage content of creatine for the animal under examination. It is convenient to employ a quantity of tissue such that when the color reaction is developed on 10 cc. of the final extract and diluted to 500 cc., the colorimetric reading on the Duboscq colorimeter will be close to 8 mm. When the final extract is made up to a volume of 200 cc., 45 grams is about the right amount of moist rabbit muscle to employ. For the cat, pig and ox—with a slightly smaller percentage content of creatine—50 grams of muscle should be employed, while for the dog, horse, man and the fowl this should be increased to 55 grams. In the case of desiccated meat, 14 grams should be employed for a dilution of 200 cc., or half that amount for a 100-cc. dilution.

<sup>4</sup> This *Journal*, xv, p. 304, 1913.

<sup>5</sup> Beker: *Zeitschr. f. physiol. Chem.*, lxxxvii, p. 21, 1913.

*Method of extraction allowing only the determination of creatine.*

The muscle sample is subjected to repeated thorough extractions with water at increasing temperatures<sup>6</sup> varying from 20°-100°C. Filtration is carried out on a funnel with gauze. Six or seven extractions with 100-cc. portions of water are made, the final extract being tested qualitatively for creatine. This will usually be found to be negative, but if an appreciable amount should be present this can be estimated and proper correction made. The united extracts are assembled in an evaporating dish, evaporated down about one-third, very carefully acidified with acetic acid to complete protein coagulation and filtered. The coagulum is thoroughly extracted to free it from creatine and added to the main filtrate. This is evaporated down to below 200 cc. and finally made up to this volume in a volumetric flask.

For the estimation of the creatine as creatinine, three 10-cc. samples of the extract are pipetted into 100-cc. Erlenmeyer flasks, 10 cc. of approximately normal hydrochloric acid added and hydrolysis carried out by the Benedict-Myers method in the autoclave at 20 pounds' pressure for twenty minutes. This method of hydrolysis is especially suitable on account of the short time required and the fact that the very light color of the extract remains unaltered. After the flasks have been removed and allowed to cool, 15 cc. of saturated picric acid and 10 cc. of 10 per cent sodium hydroxide are added, the color allowed to develop for five to eight minutes, dilution made to 500 cc., and the usual colorimetric reading made at once.

*Method of extraction allowing the determination of both creatine and creatinine in the same sample.*

The muscle sample is thoroughly extracted four or five times with water at room temperature, filtration being made each time on a funnel through gauze.<sup>7</sup> For the purpose about 500 cc., or if

<sup>6</sup> Warm water is probably not necessary since identical results are obtained by the method described below in which only water at room temperature is employed.

<sup>7</sup> At first the completeness of the creatine extraction should be controlled by qualitatively testing an additional extract as in the previous method.

necessary 600 cc., of water are employed, 25 cc. of a moderately thin alumina cream being added to the filtrate before this final volume is reached. After thorough mixing the material is filtered. Filtration takes place very rapidly. It is almost completely protein-free, and water-clear, in the case of rabbit muscle.

One-half of this (250 cc.) is employed for the estimation of the creatine, being concentrated in an 18-cm. evaporating dish over the waterbath and made up to 100 cc., *i.e.*, half the volume of the previous method. The colorimetric estimation is made as described above.

Two-fifths (200 cc.) of the filtrate is employed for the estimation of the creatinine. Half this amount may be sufficient in certain cases, *e.g.*, when the muscle has been allowed to autolyze for several weeks. The perfectly clear extract is evaporated nearly to dryness in an 18-cm. dish at room temperature with the aid of an electric fan.<sup>8</sup> Several drops of chloroform or the picric acid to be employed may be added as a preservative, although the short length of time and the low temperature due to the evaporation would appear to make this unnecessary. With perfectly fresh muscle, evaporation should be carried practically to dryness. If the material has been evaporated to dryness, 2 cc. of water are added, and, in either case, 3 cc. of saturated picric acid and 1 cc. of 10 per cent sodium hydroxide. After the color has been allowed to develop, dilution is made to 50 cc. or 100 cc. and the usual colorimetric reading made. Should 4 or 5 mgms. of creatinine be present, requiring a dilution of 200 or 250 cc., the usual quantities of picric acid and alkali can be employed, but where only 1 or 2 mgms. are present, necessitating dilutions of only 50 or 100 cc., the large amount of picric acid partially hides the depth of red color, thus giving low results.

The results which have been obtained for creatine with the two methods of extraction show perfect agreement, while for the creatinine determination, excellent duplicates have been obtained and its reliability checked in a variety of ways.

With the aid of this method we believe we have demonstrated that creatinine does exist in small amount in fresh muscle, about

<sup>8</sup> A number of extracts can be evaporated to the volume (2-10 cc.) suitable for the usual colorimetric estimation over night.

4 to 8 mgms. per 100 grams. When muscle is allowed to autolyze the creatinine increases at a very uniform rate at the expense of the creatine. The rate of the reaction gradually slows up and finally an equilibrium point is reached. Added creatine experiences the same fate as the creatine originally present, while added creatinine inhibits the reaction, or if added in sufficient quantity causes it to proceed in the opposite direction. The results of this work on tissue autolysis will be reported shortly.



# THE PARTIAL ENZYMATIC HYDROLYSIS OF YEAST NUCLEIC ACID.

By WALTER JONES AND A. E. RICHARDS.

(From the Laboratory of Physiological Chemistry, Johns Hopkins University.)

(Received for publication, January 3, 1914.)

The well known work of Kossel and his associates on the chemistry of nucleic acids culminated in the discovery that thymus nucleic acid is composed of six groups so that when it is submitted to hydrolysis with mineral acids it produces the six substances guanine, adenine, cytosine, thymine, phosphoric acid and hexose.<sup>1</sup>

Kossel also showed that while yeast nucleic acid contains groups very similar to those of thymus nucleic acid, it nevertheless gives uracil instead of thymine<sup>2</sup> and pentose instead of hexose.<sup>3</sup> A comparison of the two nucleic acids in this respect is made in the following table.

## Hydrolytic products.

Thymus nucleic acid (animal nucleic acid)	Yeast nucleic acid (plant nucleic acid)
Phosphoric acid	Phosphoric acid
Guanine	Guanine
Adenine	Adenine
Cytosine	Cytosine
Thymine	Uracil
Hexose (i.e., laevulinic acid and formic acid)	Pentose

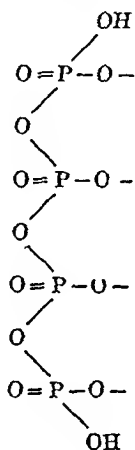
The facts embraced in this table are among the most firmly established conclusions of physiological chemistry and constitute the source from which all the more recent discussions of the subject have proceeded. They serve to characterize the two types into which nucleic acids naturally fall, for all substances of this

<sup>1</sup> Kossel and Neumann: *Ber. d. d. chem. Gesellsch.*, xxvii, p. 2215.

<sup>2</sup> Ascoli: *Zeitschr. f. physiol. Chem.*, xxxi, p. 161.

<sup>3</sup> Kossel: *Arch. f. (Anat. u.) Physiol.*, 1893, p. 157.

class have been found to yield the one or the other of these two sets of hydrolytic products. To solve the constitution of nucleic acids it therefore remained only to find the order in which the six groups are arranged. In this direction also Kossel made the first advance,<sup>4</sup> when he obtained polyphosphoric acid from yeast nucleic acid and thus furnished a strong indication that the nucleic acid has at the base of its structure some such complex as that represented by the formula:



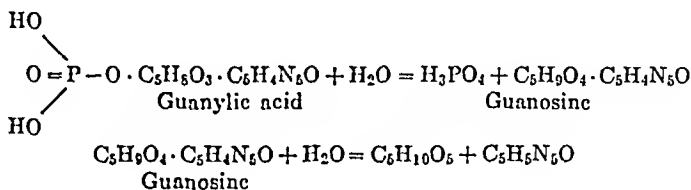
At this point the subject assumed a little complication when Ivar Bang<sup>5</sup> introduced a compound called guanylic acid, which he obtained from the pancreas and which he believed to yield on hydrolysis phosphoric acid, guanine, pentose and *glycerin*. This complication to which the discovery of guanylic acid gave rise was considerably simplified when Steudel<sup>6</sup> found that the substance does not yield glycerin on hydrolysis. But there remained and remains to the present day the anomaly that animal glands contain a substance which is chemically related to nucleic acid since it produces both phosphoric acid and guanine, but can scarcely be physiologically related to the nucleic acid of *animal* cell nuclei since it contains a pentose group, that is to say, the

<sup>4</sup> Kossel: *Zeitschr. f. physiol. Chem.*, xxxi, p. 428.

<sup>5</sup> Ivar Bang: *ibid.*, xxvi, p. 133.

<sup>6</sup> Steudel: *ibid.*, liii, p. 539.

carbohydrate group of *plant* nucleic acid. While this matter was undergoing considerable speculation it was found that guanylic acid is not confined to the pancreas, but is very widely distributed in animal glands,<sup>7</sup> and shortly afterwards Levene and Jacobs<sup>8</sup> solved its chemical constitution. By submitting the substance to neutral hydrolysis under pressure they found that it lost phosphoric acid, giving a beautifully crystalline compound called guanosine, which in turn yields guanine and pentose.



Thus guanylic acid is representative of a class of substances called *nucleotides*, i.e., compounds in which groups of phosphoric acid and nitrogenous rings are linked by carbohydrate groups.

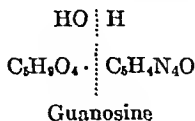
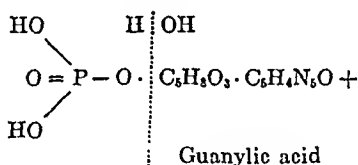
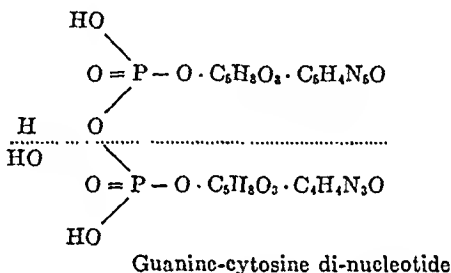
Levene and Jacobs' then applied to yeast nucleic acid the method which they had successfully employed with guanylic acid, and found that by neutral hydrolysis under pressure the substance loses phosphoric acid and yields *four* nucleosides, one of which is identical with the nucleoside guanosine, similarly obtained from guanylic acid. It would therefore appear that, as guanylic acid is a *mono-nucleotide*, yeast nucleic acid is a *tetra-nucleotide* and is composed of four *mono-nucleotide* groups as is seen in the following formula:

<sup>7</sup> Odenius: *Maly's Jahresbericht*, xxx, p. 39; Jones and Rowntree: *this Journal*, iv, p. 298; Levene and Mandel: *Biochem. Zeitschr.*, x, p. 221.

<sup>8</sup> Levene and Jacobs: *Ber. d. d. chem. Gesellsch.*, xlii, p. 2469.

<sup>9</sup> Levene and Jacobs: *ibid.*, xlii, pp. 2474 and 3247.

stances liberate one-half of their phosphoric acid by mild acid hydrolysis and each compound is thus shown to contain both a purine and a pyrimidine group. These two di-nucleotides, whose full discussion we shall defer to a future communication, are formed from yeast nucleic acid by the action of a ferment present in pig's pancreas, which should obviously be termed tetra-nuclease. Continued action of the ferments of pig's pancreas evidently decomposes the di-nucleotides into their constituent mono-nucleotides, (one of which is guanylic acid) and these in turn are converted into nucleosides from which free purine bases are finally produced.

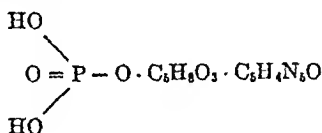


But when the extract of pig's pancreas is allowed to digest at 40° before adding the yeast nucleic acid its ferments are gradually destroyed and in an order inverse to that in which they are exerted upon nucleic acid; so that by properly selecting the duration of this preliminary digestion one should be able to gain possession of a ferment solution which will decompose a nucleic acid to a given point but no farther. In practice, however, this is exceedingly difficult to do, as the duration of digestion is not

the only factor which determines the destruction of the ferments, so that one will either allow the fresh pancreas to digest for too short a time, in which case its subsequent action on yeast nucleic acid will proceed beyond the decomposition of guanylic acid; or he will allow the fresh pancreas extract to digest for too long a time and its subsequent action on yeast nucleic acid will not proceed so far as the formation of guanylic acid.

In the majority of experiments, therefore, guanylic acid will either not be found present among the products of digestion, or will be contaminated with the guanine-cytosine di-nucleotide, since the method of isolation is the same for both substances.

We now find that guanylic acid occurs as one of the *end products* of the action of yeast on yeast nucleic acid, so that the one substance can be obtained from the other by a process which involves no judgment on the part of the experimenter and its isolation from the products is effected by a process so simple as scarcely to admit of failure. The guanylic acid thus obtained has been thoroughly identified with the guanylic acid originally found by Bang<sup>11</sup> in the pancreas. It yields sharply the quantities of nitrogen, phosphorus and guanine required for the formula,



liberates its entire phosphoric acid by mild acid hydrolysis, forms an acid potassium salt insoluble in alcohol, responds to the pentose color reaction of Tollens,<sup>12</sup> and produces the beautifully crystalline brucine salt which Levene and Jacobs<sup>13</sup> obtained with the guanylic acid of the pancreas. In short there is no observable difference between the substance which we obtained by the enzymatic decomposition of yeast nucleic acid and the pancreatic guanylic acid, whose structure Levene and Jacobs have determined. It cannot, therefore, be doubted that the molecule of yeast nucleic acid contains a guanylic acid group.

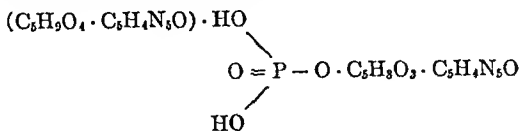
<sup>11</sup> Bang: *loc. cit.*

<sup>12</sup> Tollens: *Ber. d. d. chem. Gesellsch.*, xxix, p. 1202.

<sup>13</sup> Levene and Jacobs: *this Journal*, xii, p. 421.

## EXPERIMENTAL PART.

A mixture of 200 grams of Lebedew's commercial yeast powder, 2000 cc. of water, and 60 grams of commercial yeast nucleic acid were allowed to digest at 40° in a loosely closed vessel with enough chloroform to prevent putrefaction. In addition, a control was made with the same materials except that no nucleic acid was added. From time to time small portions of the clear fluid were withdrawn and tested for nucleic acid by acidification with sulphuric acid. The amount of nucleic acid precipitated in this way was found to diminish continually as the digestion proceeded, until the fifth day, when the failure of sulphuric acid to produce any precipitate at all with the product, showed that the nucleic acid had entirely disappeared. The yellow fluid, while still warm, was siphoned as sharply as possible from the sediment of yeast detritus and allowed to remain in an ice chest over night, when there occurred a copious deposition of flocculent material. On the contrary the fluid from the control gave no precipitate at all when cooled, showing that the substance in question originates from the nucleic acid employed and not from the yeast powder. Its amount varies somewhat in different experiments and can in any case be considerably increased by concentrating the product of digestion at 50° under diminished pressure and adding a very small amount of alcohol to the cooled fluid. The substance was filtered off in the ice chest, dissolved in boiling water, the solution filtered with a hot water funnel and again cooled in the ice chest. The deposited material was filtered, washed with alcohol and allowed to dry in a sulphuric acid desiccator. The product is a snow-white, amorphous powder, whose chemical composition is very close to that of the guanosine salt of guanylic acid.



By treatment of its solution in hot water with neutral lead acetate the lead salt of guanylic acid was precipitated. This was suspended in hot water, decomposed with sulphuretted hydrogen, and the fluid obtained after filtering off the lead sulphide was

evaporated to a small volume at  $50^{\circ}$ , under diminished pressure. After the concentrated solution had been cooled to zero guanylic acid separated, which was filtered off, washed with alcohol and allowed to dry in a vacuum over sulphuric acid. The substance thus obtained is a snow-white powder quite soluble in cold water and very easily soluble in warm water, forming a solution which does not gelatinize when cooled.

I. 0.2489 gram required 13.01 cc. of standard sulphuric acid (1 cc. = 0.003695 gram N).

II. 0.1710 gram required 8.81 cc. of the same sulphuric acid.

	Theoretical for $C_{10}H_{13}N_5O_8P$ :	Found:	
		I	II
N.....	19.23	19.36	19.10

0.380 gram of substance was heated for an hour with 10 cc. of 5 per cent sulphuric acid. While still hot the product was made alkaline with ammonia and the precipitated guanine filtered off. It weighed 0.1499 gram.

	Theoretical for guanylic acid:	Found:
Guanine.....	41.48	39.45

A drop of the filtrate from guanine failed to give a precipitate with silver nitrate in ammonia. Hence adenine was not present. The bulk of the fluid was then treated with an excess of magnesia mixture and the precipitated magnesium ammonium phosphate was filtered off and weighed. 0.380 gram gave 0.237 gram of  $NH_4MgPO_4 \cdot 6H_2O$ .

	Theoretical for guanylic acid:	Found:
Total phosphorus .....	8.79	8.14

This is of course not an accurate method of determining the phosphorus in guanylic acid but is given because it shows that *all* (not half) of the phosphoric acid of this compound is easily liberated by mild hydrolysis. Therefore the substance does not contain a pyrimidine group.

A portion of guanylic acid dissolved in water was treated with a small amount of a solution of potassium acetate and the acid potassium salt of guanylic acid was precipitated by the addition of alcohol. The total phosphorus of the substance was determined by fusion, precipitation first with molybdic solution, and then with magnesia mixture.

0.6726 gram gave 0.4120 gram  $NH_4MgPO_4 \cdot 6H_2O$ .

	Theoretical for $C_{10}H_{13}N_5O_8PK$ :	Found:
P.....	7.96	7.99

The original filtrate from lead guanylate contains guanosine, which can be easily isolated in the following way. The solution is treated with sulphuretted hydrogen filtered from lead sulphide and evaporated at  $50^{\circ}$ , under diminished pressure. The addition of neutral lead acetate to this concentrated fluid usually precipitates a small amount of guanylic acid which remained in solution after the former treatment with lead acetate, owing to the greater volume of fluid, but which must be removed at this point if a finely crystalline specimen of guanosine is to be obtained. The perfectly clear fluid is then treated alternately with small portions of lead acetate and ammonia as long as either reagent produces a precipitate and the precipitated lead compound of guanosine is suspended in hot water and decomposed with sulphuretted hydrogen. After evaporating the filtrate from lead sulphide at  $50^{\circ}$  under diminished pressure and cooling the concentrated fluid in the ice chest, guanosine is deposited in clusters of macroscopic needles, which upon recrystallization from hot water form individual long thin plates with sharp edges.

0.2176 gram required 12.82 cc. of standard sulphuric acid (1 cc. = 0.003695 gram N).

	Theoretical for $C_5H_7O_4.C_5H_7N_5O_2.2H_2O$ :	Found:
N.....	21.94	21.76
0.4374 gram of the substance lost 0.0470 gram by heating an hour at $105^{\circ}$ .		

	Theoretical for $C_5H_7O_4.C_5H_7N_5O_2.2H_2O$ :	Found:
$2H_2O$ .....	11.3	10.7

The formation of both guanosine and guanylic acid from yeast nucleic acid of course does not prove that the nucleic acid contains in its structure two guanine groups. It seems more probable that guanylic acid alone is first formed and that part of this is decomposed forming guanosine. The two substances then combine to form the compound which has been described whose physical properties make possible its easy separation from the other products of the digestion, thus furnishing a most convenient method of preparing both guanosine and guanylic acid. The same compound of guanylic acid and guanosine can be found among the products of the action of fresh pancreas extract on yeast nucleic acid; but in order to obtain the substance in quantity it is necessary to observe certain precautions which will be considered in a future contribution.

# THE OSMOTIC PROPERTIES OF THE ADDUCTOR MUSCLE OF THE CLAM—VENUS MERCENARIA.

By EDWARD B. MEIGS.

(From the Wistar Institute of Anatomy and Biology and the Marine Biological Laboratory at Woods Hole.)

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## INTRODUCTION.

It is a well-established fact that the cells of many animal and plant tissues are bounded by semi-permeable surfaces—surfaces which are more permeable to water than they are to salts and sugars in solution. This fact is particularly well established in the case of vertebrate striated muscle; and there is much further evidence for the view that the irritability of this tissue is dependent on the integrity of the semi-permeable surfaces of its fibers. So far as is known, the muscle loses its power to respond to stimuli whenever its semi-permeable properties are destroyed;<sup>1</sup> Nernst,<sup>2</sup> Höber,<sup>3</sup> and Lillie<sup>4</sup> have based interesting theories of stimulation on the view that semi-permeable surfaces play an important part in the irritability of tissues.

The evidence which has been accumulated seems sufficient to show that semi-permeable surfaces play an important part in the irritability of vertebrate striated muscle. But in regard to vertebrate smooth muscle there is already in existence a considerable body of evidence which points to the conclusion that this tissue does not possess any semi-permeable surfaces.<sup>5</sup> And even if this

<sup>1</sup> Overton: *Arch. f. d. ges. Physiol.*, xcii, p. 115, 1902; Meigs: *Amer. Journ. of Physiol.*, xxvi, p. 195, 1910; Beutner: *Biochem. Zeitschr.*, xxxix, p. 280, 1912.

<sup>2</sup> Nernst: *Göttinger Nachrichten, Mathem. physik. Klasse*, 1899, Heft 1.

<sup>3</sup> Höber: *Physikalische Chemie der Zelle und der Gewebe*, 3d edition, Leipzig, 1911, pp. 488 et seq.

<sup>4</sup> Lillie: *Pop. Sci. Monthly*, February 1913, p. 132.

<sup>5</sup> Meigs: *Journ. of Exp. Zoölogy*, xiii, p. 497, 1912.

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particular aspect of the evidence be disregarded, there still remains other independent evidence which shows that the mechanisms on which the irritability of striated and smooth muscle respectively depend must be very different from one another. Striated muscle, for instance, when immersed in isotonic solutions of non-electrolytes or of potassium salts, loses its irritability very quickly;<sup>6</sup> whereas smooth muscle remains irritable for a long time in such solutions.<sup>7</sup>

However these facts may be ultimately explained, it will evidently be dangerous to construct general theories regarding the parts played by semi-permeable membranes and by the ions of particular electrolytes in irritability until our knowledge of the osmotic properties of tissues is more complete. The following article records an attempt to gain further knowledge of the osmotic properties of the adductor muscle of the common clam, *Venus Mercenaria*.

### *Histology of the clam's adductor muscle.*

The adductor muscles of lamellibranchs consist of two portions—one white and opaque, and the other pinkish-yellow and translucent. These portions may be called, according to the nomenclature used by Marceau,<sup>8</sup> the *nacreous* and *vitreous* portions respectively. The clam has two adductor muscles, one near the anterior end, and the other near the posterior end; the nacreous portions of the muscles lie toward the periphery of the animal; and the vitreous portions, toward the center. All the experiments reported in this article were performed on the vitreous portions of the muscle. This tissue consists of elliptical or ribbon-shaped strands, which have a long diameter of about  $6\mu$  and a short diameter of about  $3\mu$ . The broad surfaces of the strands show a clearly marked double oblique striation. Careful study shows that this appearance is caused by two systems of parallel oblique striae running in diagonally opposite directions, the one on one side of the muscular strand and the other on the other side.

In cross sections of the tissue the strands are seen to be divided rather irregularly into groups by thin membranes which to some

<sup>6</sup> Overton: *Arch. f. d. ges. Physiol.*, xcii, p. 346, 1902.

<sup>7</sup> Meigs: *Journ. of Exp. Zoölogy*, xiii, p. 497, 1912.

<sup>8</sup> Marceau: *Arch. de zoologie exp. et gén.*, 5 serie, ii, p. 295, 1909.

extent resemble the sarcolemmas of vertebrate striated muscle. In the vertebrate muscle, however, each fiber has its separate sarcolemma which completely encloses it; while the membranes of the clam's muscle do not completely enclose any particular portions of it, but appear in cross sections as branching lines, which run up to and apparently form connections with that portion of the mantle which covers the surface of the muscle: there is no division of the muscle substance by the membranes into distinct and separate fibers. For further details regarding the histology of this form of muscle the reader is referred to the article by Marceau (1909) which was quoted above.

*The chlorine content of the clam's adductor muscle, and of the fluid medium by which it is surrounded.*

A very significant fact in the physiology of the types of muscle which have hitherto been studied is the great difference in the chemical composition of the ash of the muscle and of the blood plasma or lymph by which the muscle is continually being washed. There is reason to believe that the striated muscle fibers of the frog contain less than one-tenth as much sodium and chlorine as the blood plasma of the same animal.<sup>9</sup>

The amount of chlorine contained in the fresh adductor muscle of *Venus* has been determined. Pieces of the muscle were dried carefully on filter-paper and fused in a platinum crucible with sodium peroxide; the amount of chlorine in the residue was then determined by the Volhard-Arnold method.<sup>10</sup> It was found that the muscle contained on the average only 0.32 per cent of chlorine—see experiments 8 and 11.<sup>11</sup>

When a living clam is broken open there can always be obtained from it a considerable quantity of "clam-juice." This fluid may be regarded as to some extent analogous to the lymph of higher animals; at any rate the adductor muscles come into close rela-

<sup>9</sup> Urano: *Zeitschr. f. Biol.*, 1, p. 212, 1907; li, p. 483, 1908; Fahr: *ibid.*, lii, p. 72, 1908.

<sup>10</sup> Hawk: *Practical Physiological Chemistry*, 3d edition, 1910, pp. 390, 391.

<sup>11</sup> Protocols of these and other experiments mentioned in the text will be found at the end of the article.

tion with it throughout the life of the clam. Garrey has determined the freezing point of "clam-juice" and finds that it is practically the same as that of sea-water;<sup>12</sup> and Griffith reports that sodium and chlorine make up about 70 per cent of the ash of lamellibranch blood.<sup>13</sup> I have carried out corresponding experiments on the chlorine contents of sea-water and clam-juice and find that there is little difference in the two cases. The average concentration of chlorine in the clam-juice was 1.67 per cent while that of sea-water in the neighborhood of Woods Hole is 1.78 per cent—see experiments 10 and 14.

It seems, therefore, that in the case of the clam as in that of vertebrates, the surrounding medium contains several times as high a concentration of chlorine as the muscle and one might be led to expect that the clam's muscle would behave in isotonic and non-isotonic solutions of sugars and salts like a tissue whose cells were bounded by semi-permeable surfaces.

*The changes of weight undergone by clam's muscle in various solutions.*

Pieces of clam's adductor muscle were cut loose from their attachments to the shell and were split into small pieces. These pieces were weighed, immersed in the solutions whose effects were to be studied, taken out at intervals, dried on filter paper, and weighed. Care was taken that each piece of muscle was dried to the same extent before each of the various weighings to which it was subjected. In all cases it was determined whether or not the muscle was irritable to a moderately strong faradic current at the end of the experiment—sometimes at other periods throughout the experiment.

Figure 1, shows the curve of change of weight undergone by the muscle in distilled water; for comparison the curve of swelling undergone by a frog's sartorius in distilled water is shown in the figure by a dotted line. It would be difficult to suppose that the two processes represented by the curves of figure 1 had anything in common. Sea-water has, of course, a much higher osmotic pressure than Ringer's solution, and if the osmotic pressure of the surroundings is balanced in the two cases by that of the muscle

<sup>12</sup> Garrey: *Biol. Bull.*, viii, p. 259, 1905.

<sup>13</sup> See v. Fürth: *Vergleichende chemische Physiologie der niederen Tiere*, Jena, 1903, p. 73.

fluids, we should expect the osmotic pressure to be much higher in the case of the clam's muscle than in that of the frog. The clam's muscle ought, therefore, to swell more rapidly in distilled water, but just the opposite is the case.<sup>14</sup> Further the early stages of the swelling of the clam's muscle have none of the characteristics of an osmotic process. But experiments with distilled water are open to the objection that muscle is killed soon after its immersion in the fluid.

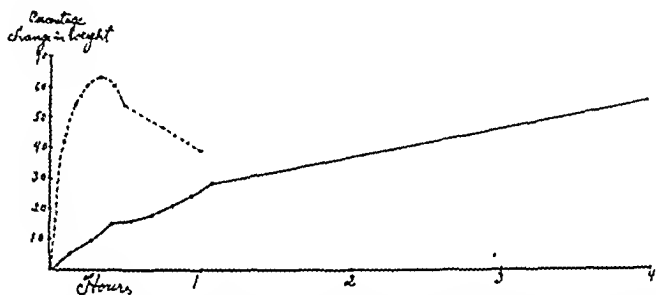


FIG. 1. Changes of weight undergone by the frog's sartorius (broken line) and by the adductor muscle of Venus (unbroken line) in distilled water. See experiment 1. The curve of swelling of frog's muscle is obtained from Meigs: *The Journal of Experimental Zoölogy*, xiii, p. 549, 1912, experiment 2.

Figure 2 shows how clam's muscle and frog's striated muscle change in weight in half-strength sea-water and in half-strength Ringer's solution respectively. In both cases the tissue is immersed in a fluid which contains all the salts in half the concentration which is supposedly physiological for it. Figure 3 shows the changes in weight undergone by the two kinds of tissue in double strength sea-water<sup>15</sup> and in double strength Ringer's solution respectively. Clam's muscle remains irritable for many hours in half-strength and in double strength sea-water, as does frog's muscle in half-strength and in double strength Ringer's solution.

<sup>14</sup> The sartorius from which the curve of figure 1 was obtained was larger than the piece of clam's muscle.

<sup>15</sup> The double strength sea-water was prepared by evaporating sea-water to half its original volume.

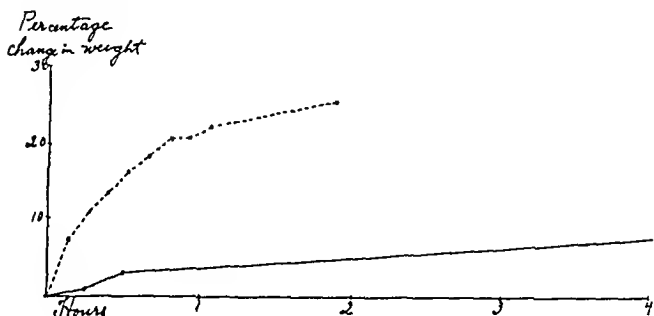


FIG. 2. Changes of weight undergone by the frog's sartorius in half-strength Ringer's solution (broken line) and by the adductor muscle of Venus in half-strength sea-water (unbroken line). See experiment 2. The curve of swelling of the frog's muscle is obtained from Meigs: *The Journal of Experimental Zoölogy*, xiii, p. 564, 1912, experiment 56.

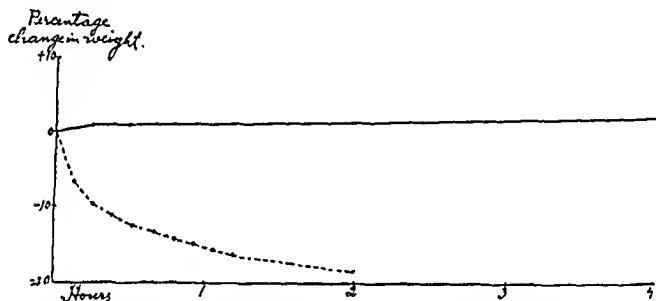


FIG. 3. Changes of weight undergone by the frog's sartorius in double strength Ringer's solution (broken line) and by the adductor muscle of Venus in double strength sea-water (unbroken line). See experiment 3. The curve of change in weight of the frog's muscle is obtained from Meigs: *The Journal of Experimental Zoölogy*, xiii, p. 563, 1912, experiment 54.

The most interesting of the results of these experiments is that the clam's muscle shows at no period any loss of weight in the double strength sea-water. This result comes near to proving that no portions of the preparation used in the experiment are surrounded by semi-permeable surfaces; for even with the most leaky semi-permeable membranes there is a considerable temporary tendency for fluid to pass from one side to the other when the salt concentration on one side is double what it is on the other.

All the other aspects of the results tell against the view that any portions of the clam's muscle are bounded by semi-permeable surfaces, but it is not necessary to dwell on these, as the points at issue have already been rather fully considered elsewhere.<sup>16</sup>

The changes of weight undergone by the adductor muscle of Venus in sea-water, in 10 per cent NaCl solution, and in 30 per cent cane sugar solution have been followed (see experiments 4, 5, 17 and 18). The muscle swells slowly in all of these solutions. It is rapidly killed in the NaCl solution, but maintains its irritability almost unimpaired for twenty-four hours or more in the sea-water and in the sugar solution. Curiously enough the swelling in sea-water in these particular experiments was at first more rapid than in half-strength sea-water. All these results speak against the view that any portions of the preparations of muscle used are surrounded by semi-permeable surfaces.

*The diffusion of chlorine out of the clam's adductor muscle into a surrounding sugar solution.*

Pieces of muscle were immersed for about forty-five hours in 30 per cent saccharose solution, and their chlorine content was then compared with that of fresh pieces of muscle. It was found that while the fresh muscle contained about 0.3 per cent of chlorine, that which had been in the sugar solution contained none. The muscle was still highly irritable after it had been for forty-five hours in the sugar solution (see experiments 8 and 9).

*The diffusion of salt and sugar into the adductor muscle of Venus from surrounding solutions.*

The fact that the clam's muscle maintains its irritability for twenty-four hours or more when immersed in 30 per cent cane sugar solution or in sea-water or double strength sea-water makes it easy to determine to what extent salt and sugar diffuse from these solutions into the living muscle.

Equal or nearly equal portions of clam's muscle were prepared in double sets. One of the portions was fused immediately with sodium peroxide and analyzed for chlorine as described above. The other portion was kept

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<sup>16</sup> Meigs: *Journ. of Exp. Zoölogy*, xiii, p. 497, 1912.

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for twenty-four hours in sea-water or double strength sea-water, tested for its irritability to the faradic current, and then analyzed for chlorine.

The amount of sugar which diffuses into the muscle in seventeen hours from a 30 per cent sugar solution was estimated by comparing the dry weight of the fresh muscle with that of muscle which had been for seventeen hours in the sugar solution. In both cases the tissue was dried for seventeen days at 98° and then for twenty-four hours at 110°. The tissue never lost more than 3 mgm. in weight after the first seventy-two hours of drying at 98°.

Fresh muscle contains on the average 0.32 per cent of chlorine, while muscle which has been for twenty-four hours in sea-water contains 0.84 per cent of chlorine; and muscle which has been for twenty-four hours in double strength sea-water, 1.68 per cent of chlorine (see experiments 11, 12 and 13). It would seem therefore that considerable quantities of chlorine diffuse into the muscle from the solutions used in the course of twenty-four hours. It is interesting to note that after twenty-four hours in either sea-water or double strength sea-water the muscle contains just about half as high a concentration of chlorine as the fluid in which it was immersed.

The detailed results of the experiments on the diffusion of cane sugar into clam's muscle are given in the experimental protocols Nos. 6 and 7. It was found that in the course of seventeen and one-half hours enough sugar diffused into the muscle from a 30 per cent saccharose solution to bring the concentration of the saccharose in the water of the muscle up to 17.5 per cent. These experiments were controlled by an experiment in which the increase in specific gravity undergone by muscle as the result of immersion in sugar solution was ascertained. It is easy to get an approximate idea of the specific gravity of a piece of clam's muscle by noting whether it sinks or floats in each of a series of sugar solutions of known specific gravities. In this way it was ascertained that the specific gravity of a piece of the muscle immersed for several hours in 30 per cent cane sugar solution increases from 1.072 to 1.096. From this it may be calculated that about 15 per cent of the weight of the water of the muscle in sugar has diffused into it. This experiment is much rougher than the other, but is free from certain objections to which the other might be considered open, and therefore serves to confirm it.

*Explanation of the low chlorine content in fresh muscle.*

It seems at first sight difficult to reconcile the results reported in the later sections of this article with the fact that clam's muscle contains under normal conditions so much less chlorine than its surroundings. Irritable preparations of the tissue show no sign of semi-permeability with respect to sugars and salts dissolved in water, and are certainly quite freely permeated by both sugars and salts; and yet the fresh muscle as obtained from the intact animal contains less than one-fifth as much chlorine as sea-water or as the "clam-juice" with which it comes into close relations.

In attempting to explain this seeming contradiction certain facts must be kept in mind. The adductor muscles of Venus are short thick masses of tissue attached at both ends to the shells, and their longitudinal surfaces are covered by layers of the mantle which consist of surface layers of epithelium with underlying connective tissue. The pieces of muscle used in the experiments on diffusion and osmosis were cut free from their attachments to the shell and stripped of mantle; it is quite possible that this last-named structure prevents the diffusion of chlorine into the muscle under normal circumstances.

Experiments were carried out to determine this point. Clams were broken open in such a way that the adductor muscles were not detached from the shells or otherwise disturbed; all soft parts of the animals except the adductor muscles were then removed; and the longitudinal surfaces of the muscles were in some cases left covered by the mantle, and in other cases scraped free of mantle. The preparations were then left for either twenty-four or forty-eight hours in running sea-water and finally analyzed for chlorine as described above. It was found that after twenty-four hours' exposure to running sea-water the muscle which had been left covered by its mantle contained 0.12 per cent of chlorine, while that which had been scraped free of mantle contained 0.34 per cent of chlorine. After forty-eight hours' exposure to the running sea-water the tissue covered by mantle contained 0.16 per cent of chlorine, while that scraped free of mantle contained 0.42 per cent of chlorine. In all cases the muscle remained irritable throughout the course of the experiments (see experiments 15 and 16).

These experiments point clearly to the conclusion that the mantle plays an important part in keeping the muscle of the normal living clam nearly free from chlorine.

It will be noted that the muscle covered by mantle and exposed to running sea-water contained decidedly less chlorine than the muscle analyzed fresh in earlier experiments. A possible explanation of this difference is the fact that the earlier experiments were carried out in July; and the others, late in September. Another point which needs a word of comment is the fact that the muscle which was scraped free of mantle and exposed for forty-eight hours to running sea-water contained only half as much chlorine as the muscle which was cut loose from its attachments to the shell and left for only twenty-four hours in still sea-water. I believe that this is explained by the following considerations. The muscle cut loose from its attachments to the shell was teased into comparatively small pieces and had therefore a decidedly larger surface in relation to its volume exposed to the fluid in which it was immersed. Further, in the running sea-water it was impossible to prevent the formation of bubbles on the surface of the tissue, and these must have interfered to a considerable extent with the diffusion of salts.

It was possible to determine roughly by inspection what portion of the muscle scraped free of mantle and exposed to running sea-water had been penetrated by the foreign salts. Clam's muscle into which chlorine has diffused appears more whitish than fresh muscle, and has less tendency to contract as a result of cutting, though it often contracts more actively in response to stimulation by the electric current. In the muscle which had been scraped free of mantle and exposed for forty-eight hours to running sea-water only that portion of the tissue (amounting to a little more than half) which lay nearest to the exposed surface had the appearance in question; the layers lying furthest from the exposed surface had the appearance of fresh muscle.

### *General discussion.*

The results of the experiments given in the foregoing pages may be summed up as follows. The whole adductor muscle of the clam is surrounded by a membrane (the mantle) which is

nearly if not quite impermeable to sodium chloride. From behind this membrane the muscle carries on its necessary interchanges with the exterior; and in some way which is still unknown the sodium chloride content of the water within the tissue is kept far below that of the exterior. If, however, the mantle be stripped away, sodium chloride diffuses in quite readily, though its concentration in the water of the muscle does not reach that of the surrounding fluid. The irritability of the muscle is not seriously interfered with by the stripping away of its mantle and the penetration of sodium chloride.

*The diffusion of crystalloids through the clam's adductor muscle.*

The amount of chlorine or sugar which a small piece of adductor muscle will take up when immersed in sea-water, double strength sea-water, or 30 per cent saccharose solution bears a definite relation to the concentration of the sodium chloride or sugar in the solution. In all my experiments the concentration of the crystalloid in the muscle rose to about half that of the surrounding solution. Clam's muscle contains about 80 per cent water and 20 per cent solids and it may therefore be said that the concentration of crystalloid in the water of the muscle reaches about  $50 \times \frac{100}{80}$  or 62.5 per cent of the concentration which it has in the fluid in which the muscle is immersed. If it be supposed that the crystalloid diffuses into one portion of the muscle's water and not into the other portion, it may be said that the concentration of the crystalloid reaches that of the exterior solution in about 62.5 per cent of the muscle's water.

These results are very similar to others which have been obtained with vertebrate smooth muscle<sup>17</sup> and it is an interesting question how the crystalloid is prevented from diffusing into so large a proportion of the water contained in the muscle. It is not a tenable hypothesis in the case of either kind of muscle that certain elements of the tissue are protected from the crystalloid by semi-permeable surfaces. This hypothesis has already been discussed for the case of the vertebrate muscle;<sup>18</sup> in the case of the clam's muscle it is made untenable by the facts that the tissue

<sup>17</sup> Meigs: *Journ. of Exp. Zoölogy*, xiii, pp. 529-31, 1912.

<sup>18</sup> Meigs: *ibid.*, xiii, pp. 540-41.

shows at no time any tendency to lose weight in double strength sea-water and that it tends to gain weight in 10 per cent sodium chloride solution. How then is the fact that the crystalloid does not reach the same concentration in the water of the muscle that it has in the surrounding solution to be explained?

To explain certain results which he has obtained with striated muscle, Overton<sup>19</sup> has advanced the hypothesis that a portion of the water contained in animal tissues is combined with the colloids in such a way that it cannot act as a solvent for salts. This hypothesis or some more or less similar one is necessary to explain the phenomena exhibited by vertebrate smooth muscle;<sup>20</sup> and from the results which have been described it is evident that a similar hypothesis is necessary in the case of the adductor muscle of Venus.

It is of course a far from complete and satisfactory explanation of the phenomena in question to say that a part of the water in animal tissues is held in more or less firm combination with the colloids. Such a statement is at best merely a rough outline of the conditions to be studied; and before our knowledge of the subject can be at all satisfactory it will be necessary to discover under what conditions colloids are capable of holding water combined in this way, what factors are capable of freeing the water from its combination, etc. These questions are more likely to be answered by a study of non-living colloids than by that of living tissues, and some experiments bearing on this side of the subject have already been reported.

Overton,<sup>21</sup> for instance, quotes certain experiments by Ludwig and Gunning which show that dried colloids immersed in salt solutions are capable of taking up water and leaving the salts to a large extent if not entirely behind. And Hofmeister<sup>22</sup> has carried out a number of experiments in which he determined the swelling undergone by gelatin in various salt solutions and the extent to which the salt diffused into the water contained in the gelatin. He states that if an experiment is carried far enough the concentration of salt in the water contained in the gelatin

<sup>19</sup> Overton: *Arch. f. d. ges. Physiol.*, xcii, pp. 128-42, 1902.

<sup>20</sup> Meigs: *Journ. of Exp. Zoölogy*, xiii, pp. 529-31, 1912.

<sup>21</sup> Overton: *Arch. f. d. ges. Physiol.*, xcii, pp. 176, 177, 1902.

<sup>22</sup> Hofmeister: *Arch. f. exp. Path. u. Pharm.*, xxviii, p. 210, 1891.

becomes nearly the same as that of the surrounding solution. His experimental figures indicate, however, that the rapidity with which the salt diffuses into the gelatin is very variable and may be very small; in one of the experiments with sodium chloride for instance (experiment 1, pages 216 and 217) the concentration of salt in the water contained in the gelatin reached only from 55 to 79 per cent of that in the surrounding solution in the course of forty-eight hours. A more or less similar result was obtained with sodium tartrate (experiment 1, pages 220 and 221). The non-living colloids are being very much studied at the present time, and it will be interesting to see how closely analogous their reactions are to those which are exhibited by muscle.

*The bearing of the results given above on theories connecting irritability with the presence of semi-permeable membranes.*

It is of course evident that the mantle which covers the surface of the clam's adductor muscle can play no essential part in the irritability of the tissue. And all the evidence which has been gathered points to the view that the irritability of the clam's muscle does not depend, like that of vertebrate striated muscle, on the presence of semi-permeable membranes with an easily disturbed arrangement of the ions of different salts on opposite sides of them. The case of clam's muscle is like that of vertebrate smooth muscle. Pieces of the former tissue, which are still highly irritable, show no tendency to lose weight in either double strength sea-water or 10 per cent sodium chloride solution, and it is therefore extremely difficult if not impossible to suppose that any portions of the irritable muscle are surrounded by semi-permeable surfaces. But even if this aspect of the evidence be disregarded, it must still be admitted that the irritability of the vertebrate striated muscle depends on entirely different conditions from that of the clam's muscle, for the latter maintains its irritability in sugar solutions after all its sodium chloride has diffused out, whereas the irritability of the former has been shown by Overton to disappear whenever any considerable portion of the sodium chloride in its interstitial spaces is replaced by any non-electrolyte and by most electrolytes.<sup>23</sup>

<sup>23</sup> Overton: *Arch. f. d. ges. Physiol.*, xcii, p. 346, 1902.

## SUMMARY.

1. The adductor muscle of Venus contains only 0.3 per cent of chlorine, although the external medium contains about 1.6 per cent of that element.

2. Chlorine is prevented from diffusing into the muscle from the exterior by the mantle, which covers its surface and is nearly if not entirely impermeable to sodium chloride.

3. The muscular elements of the adductor are not surrounded by semi-permeable membranes. This is shown by the facts that small pieces of the muscle, which remain irritable for a long time under all sorts of conditions, take up considerable quantities of chlorine from sea-water, and fail to lose weight in double strength sea-water and in 10 per cent NaCl solution.

4. In the terms of Overton's hypothesis, about 38 per cent of the water normally present in the adductor muscle is combined with the colloids of the tissue in such a way that it cannot act as a solvent for sugars or salts. This condition corresponds quite closely with what is found to be the case in vertebrate smooth muscle.

5. The conditions determining the irritability of the adductor muscle of Venus to electric currents are fundamentally different from those which obtain in the case of vertebrate striated muscle.

## PROTOCOLS OF THE EXPERIMENTS.

All of the following experiments were performed on the vitreous portions of the adductor muscle.

EXPERIMENT 1. *May 21, 1912.* Piece of adductor muscle of Venus weighed fresh 0.089 gram. Temperature throughout experiment remained at 26°.

11.04 a.m., immersed in distilled water.

Time.	11.12	11.20	11.23	11.35	11.44	11.52	12.00	12.03	3.04
Weight....	0.093	0.097	0.102	0.103	0.105	0.108	0.110	0.114	0.138

At 3.04 p.m. entirely unirritable.

EXPERIMENT 2. *October 15, 1912.* Piece of adductor muscle of Venus weighed fresh 0.201 gram. Temperature varied between 15 and 21°. This muscle came from the same animal as that of experiment 3.

4.15 p.m., immersed in half-strength sea-water.

Time.	4.30 p.m.	4.45 p.m.	9.15 a.m. Oct. 16.
Weight....	0.202 gram.	0.207 gram.	0.257 gram.

Still quite irritable at 9.15 a.m., October 16.

EXPERIMENT 3. *October 15, 1912.* Piece of adductor muscle of Venus weighed fresh 0.116 gram. Temperature varied between 15 and 21°. This muscle came from the same animal as that of experiment 2.

4.20, p.m. immersed in double strength sea-water.

Time.	4.35 p.m.	4.50 p.m.	9.20 a.m. Oct. 16.
Weight....	0.117 gram.	0.117 gram.	0.125 gram.

Still quite irritable at 9.20 a.m., October 16.

EXPERIMENT 4. *October 17, 1912.* Piece of adductor muscle of Venus weighed fresh 0.204 gram. Temperature in the experiment varied between 20 and 21°. This muscle came from the same animal as that of experiment 5.

11.15 a.m., immersed in sea-water.

Time.	11.30	12.15	1.15	2.15	3.15	4.15	9.30 a.m. Oct. 18.
Weight....	0.211	0.217	0.224	0.229	0.231	0.233	0.230 gram.

Still somewhat irritable at 9.30 a.m., October 18.

EXPERIMENT 5. *October 17, 1912.* Piece of adductor muscle of Venus weighed fresh 0.196 gram. Temperature in this experiment varied between 20 and 21°. This muscle came from the same animal as that of experiment 4.

11.20 a.m. immersed in 30 per cent cane sugar solution.

Time.	11.35	12.20	1.20	2.20	3.20	4.20	9.35 a.m. Oct. 18.
Weight....	0.195	0.210	0.222	0.231	0.236	0.240	0.286 gram.

Still highly irritable at 9.35 a.m., October 18.

EXPERIMENT 6. *March 18, 1913.* Piece of adductor muscle of Venus weighed fresh 0.2325 gram. This muscle came from the same animal as that of experiment 7.

4.10 p.m., placed in oven at 98° and dried for fifteen days at that temperature.

11.10 a.m., March 28, weighed 0.0456 gram; placed in oven at 110° and dried at that temperature for one day.

11.10 a.m., March 29, weighed 0.0454 gram.

This muscle contained therefore 80.5 per cent of water and 19.5 per cent of solids.

EXPERIMENT 7. *March 13, 1913.* Piece of adductor muscle of Venus weighed fresh 0.2572 gram. This muscle came from the same animal as that of experiment 6.

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5.30 p.m., immersed in 30 per cent cane sugar solution at temperature of 8°.

9.45 a.m., March 14, temperature of sugar solution, 9°.

11.00 a.m., muscle weighed 0.3183 gram; still highly irritable; placed in oven at 98° and dried for fourteen days at that temperature.

11.00 a.m., March 28, weighed 0.0903 gram. Placed in oven at temperature of 110° and dried for one day.

11.00 a.m., March 29, weighed 0.0901 gram.

The adductor muscle of experiment 7 weighed fresh 0.2572 gram, and of this, according to experiment 6, 19.5 per cent or 0.0501 gram was composed of solids. Therefore, 0.0901—0.0501 or 0.04 gram in sugar may be supposed to have diffused into the muscle of experiment 7 in the course of seventeen and one-half hours. Muscle after stay in sugar solution weighed 0.3183 gram. Of this 0.3183—0.0901 or 0.2282 gram was water. That is, total water of this muscle was made up to 17.5 per cent sugar solution or it may be said that about 58 per cent of water of muscle was made up to 30 per cent sugar solution.

EXPERIMENT 8. *July 3, 1913.* Portion of adductor muscle of Venus weighing fresh 3.326 grams was fused with  $\text{Na}_2\text{O}_2$  in platinum and analyzed for Cl. 1.58 cc. standard  $\text{AgNO}_3$  (1 cc. = 0.006 gram Cl) were required.  $\text{Cl} = 0.00948 \text{ gram} = 0.28 \text{ per cent}$ . (Other similar determinations yielded 0.32, 0.34 and 0.35 per cent respectively. Average of all = 0.32 per cent.)

EXPERIMENT 9. *July 3, 1913.* From the animal used in experiment 8 a portion of adductor muscle weighing fresh 3.095 grams was taken and at 11.25 a.m. immersed in 30 per cent cane sugar at 17°.

9.00 a.m. July 5, weight, 3.840 grams: still quite irritable. During this interval the sugar solution was changed four times, temperature varying between 11° and 17°. Analysis at end of this time showed the absence of Cl. This result was confirmed in two similar experiments.

EXPERIMENT 10. *July 9, 1913.* Ten cc. of sea-water from the neighborhood of Woods Hole analyzed for Cl required 29.72 cc. standard  $\text{AgNO}_3$  solution.  $\text{Cl} = 0.1783 \text{ gram} = 1.783 \text{ per cent}$ .

EXPERIMENT 11. *July 10, 1913.* Portion of adductor muscle of Venus weighing fresh 2.970 grams was fused with  $\text{Na}_2\text{O}_2$  in platinum and analyzed for Cl. Required 1.6 cc. standard  $\text{AgNO}_3$ .  $\text{Cl} = 0.0096 \text{ gram} = 0.32 \text{ per cent}$ .

EXPERIMENT 12. *July 10, 1913.* From the animal used in experiment 11 a portion of adductor muscle weighing fresh 2.515 grams was taken and at 11.40 a.m. immersed in sea-water at 15°. At 9 a.m., July 11, it was fairly irritable and weighed 2.641 grams. Temperature during the interval was 14–15°.

On analysis for Cl, 3.53 cc. standard  $\text{AgNO}_3$  were required.  $\text{Cl} = 0.02118 \text{ gram} = 0.842 \text{ per cent}$ .

EXPERIMENT 13. *July 10, 1913.* From the animal used in experiments 11 and 12 a portion of adductor muscle weighing fresh 2.765 grams was immersed at 11.50 a.m. in double strength sea-water at a temperature of 15°. At 11.45 a.m. July 11, it weighed 2.597 grams, the temperature during the interval varying from 14-15°. A sample immersed with it had lost its irritability but regained it completely when immersed for about five hours in sea-water at a temperature of 14°.

On analysis for Cl, 7.77 cc. standard  $\text{AgNO}_3$  were required.  $\text{Cl} = 0.0466$  gram = 1.68 per cent.

EXPERIMENT 14. *July 14, 1913.* "Clam juice" (which contains a small amount of organic matter) was analyzed for Cl. 4.2965 grams required 11.9 cc. standard  $\text{AgNO}_3$ .  $\text{Cl} = 0.0714$  gram = 1.66 per cent. In another analysis,  $\text{Cl} = 1.67$  per cent.

EXPERIMENT 15. *September 25, 1913.* The adductor muscles from six clams left *in situ* attached to their shells and covered by mantles, but the soft parts dissected away, were immersed in running sea-water at 8.30-10.30 a.m. The temperature of the latter varied from 18-19° between this time and 9.00 a.m., September 27, when a small sample was still slightly irritable. 6.553 grams muscle fused with  $\text{Na}_2\text{O}_2$  in platinum on analysis for Cl required 1.8 cc.  $\text{AgNO}_3$  solution.  $\text{Cl} = 0.0108$  gram = 0.16 per cent.

EXPERIMENT 16. *September 15, 1913.* Adductor muscles of six clams<sup>24</sup> left *in situ* in shells, but mantles as well as soft parts dissected away, were immersed in running sea-water at 8.30-10.30 a.m. At 9.00 a.m., September 27, temperature remaining 18-19°, a small piece was still somewhat irritable. On analysis for Cl, 7.313 grams required 5.1 cc. standard  $\text{AgNO}_3$  solution.  $\text{Cl} = 0.0306$  gram = 0.42 per cent.

(In two other similar experiments except that the muscles were left for only twenty-four hours instead of forty-eight hours in running sea-water, the portion of tissue left covered by mantle was found to contain 0.12 per cent Cl, while that from which the mantle was dissected, contained 0.34 per cent.)

EXPERIMENT 17. *November 21, 1913.* Piece of adductor muscle of Venus weighed fresh 0.260 gram. Temperature varied between 18 and 21°.

3.25 p.m., immersed in 10 per cent  $\text{NaCl}$  solution.

Time.	3.30	3.35	3.40	3.45	3.50	3.55	4.10	4.25	9.15 Nov. 22.
Weight...	0.268	0.271	0.272	0.273	0.274	0.275	0.278	0.278	0.292 gram.

Three strips were torn from surface of this piece of muscle; one, stimulated immediately, was found entirely unirritable; the other two were immersed for three hours in sea-water and at the end of that time were still found to be entirely unirritable.

<sup>24</sup> The clams used in experiments 15 and 16 were the same. In three of the animals the anterior muscle was left covered by mantle, and in the others, the posterior.

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EXPERIMENT 18. *November 21, 1913.* Two small portions of the adductor muscle of Venus, weighing respectively 0.033 and 0.066 gram, immersed for an hour in 10 per cent NaCl solution, were found at the end of this period to be entirely unirritable. Both pieces were then transferred to sea-water and after seventeen hours in that fluid were found to be somewhat irritable. The temperature varied between 18 and 21°.

# A METHOD FOR THE DETERMINATION OF THE TOTAL FATS OF UNDRIED FECES AND OTHER MOIST MASSES.

By GORDON J. SAXON.<sup>1</sup>

(From the *Pepper Laboratory of Clinical Medicine, University of Pennsylvania.*)

(Received for publication, January 3, 1914.)

The procedure about to be detailed is essentially a combination of the excellent method of Folin and Wentworth<sup>2</sup> for the determination of the total fats of powdered dried feces, and the method of Meigs<sup>3</sup> for the determination of the fat of milk.

It is as follows: In the case of feces the twenty-four-hour specimen is collected and very thoroughly mixed until a homogeneous paste results. If the stool is liquid, infusorial earth is slowly added while thoroughly mixing, until a workable paste results. The mass is weighed before and after a sample for extraction is chosen. If the mixing has been done in an ordinary mortar it is advisable to remove the mass quantitatively, and place it on waxed paper for weighing.

The sample for extraction is placed in a 100-cc. glass stoppered, graduated cylinder. Care must be taken not to smear the neck of the cylinder. This may be avoided by removing from various portions of the mass small bits with the aid of short capillary tubes sealed at both ends. The tubes and the portions of specimen which they carry are dropped into the cylinder. The specimen may be as much as 5-6 grams.

Add 20 cc. of distilled water and 1-2.5 cc. of concentrated hydrochloric acid (depending on amount of sample) and again sufficient

<sup>1</sup> Woodward Fellow in Physiological Chemistry.

<sup>2</sup> Folin and Wentworth: *this Journal*, vii, p. 421, 1910.

<sup>3</sup> Arthur V. Meigs: *Phila. Med. Times*, July 1, 1882; Arthur V. Meigs and Howard L. Marsh: *The Medical Record*, Dec. 30, 1911; Croll: *Biochem. Bull.* for June, 1913.

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water to make a total bulk of 30 cc. Add exactly 20 cc. of ether and shake vigorously for five minutes. Allow to stand a few seconds, remove stopper and add exactly 20 cc. of 95 per cent alcohol and again shake for five minutes.

Stand the cylinder aside. The ether containing practically all of the fat will come to the top as a colored transparent layer. The ether layer is blown off into a tall 150-200 cc. beaker. This is accomplished in the same manner that water is blown from a wash bottle. The submerged end of the delivery tube is bent upward as in the pipette described by Meigs and Marsh<sup>4</sup> in order that there be no upward currents which would set in motion the subjacent alcohol-water-feces layer. In this manner practically all the ether can be removed. The thin layer which remains is diluted with 5 cc. of ether, slightly agitated and blown off. This is done in all five times, care being taken each time to wash down the sides of the cylinder. The stopper also should be washed.

Twenty cc. of ether are again added, and the cylinder shaken for five minutes and set aside. When the ether has nearly stratified blow it off and wash as before. During the second washing process the stratification will complete itself. Evaporation is carried on until no trace of alcohol which has been carried over by the ether remains. From this point on the method is that of Folin and Wentworth. To the residue add 30 cc. of low-boiling petroleum ether (should distil over below 60°C.) and allow to stand over night. Petroleum ether for this work should be frequently tested for a residue on evaporation. If a residue is left the ether should be redistilled.

Filter the fatty petroleum ether, catch filtrate and washings in a tall, weighed, 100-cc. beaker, evaporate off the solvent, dry the beaker at 90°C., desiccate and weigh. Subtract the weight of the beaker from the last weighing and the result is the weight of neutral fat, free fatty acids and the fatty acids of the soaps contained in the specimen extracted.

The fatty acid titre is obtained by dissolving the contents of the beaker after the weighing just mentioned in 50 cc. of benzol, heating almost to the boiling point, adding two drops of a 0.5 per cent alcoholic solution of phenolphthalein and titrating with a decinor-

<sup>4</sup> This *Journal*, xvi, p. 152, 1913.

mal solution of sodium alcoholate. Each cubic centimeter of the standard solution used in the titration represents 28.4 mgm. of stearic acid.

The difference between the gravimetric and the volumetric determinations is the weight of the neutral fat. In the preparation of the sodium alcoholate solution absolute alcohol and freshly cut bright metallic sodium are used; otherwise it is the same as the standardization and preparation of any other standard solution.

Following are determinations showing the accuracy of the method: Three specimens of stearic acid were melted, mixed with infusorial earth and extracted by the wet method with the following results:

0.0830 gram stearic acid used..... 0.0828 gram recovered.  
 0.0970 gram stearic acid used..... 0.0964 gram recovered.  
 0.1632 gram stearic acid used..... 0.1622 gram recovered.

In order that a comparison might be made between this method and that of Folin and Wentworth, a dried, powdered stool was used to which infusorial earth had been added previous to drying in sufficient quantity to render workable the specimen, which was large and semi-liquid when it came to the laboratory.

TABLE I.

WEIGHT OF SPECIMEN	WEIGHT OF TOTAL FATS	FATTY ACID TITRE IN GRAMS	PERCENTAGE OF TOTAL FATS
0.5	0.0306	0.017	6.12
0.5	0.0301	0.017	6.02
0.5	0.0306	0.017	6.12
0.5	0.0311	0.017	6.22

Average of four determinations, 6.12 per cent.

This table shows the results of four determinations by the wet method.

The next table shows results by the Folin-Wentworth method. Under the heading "Re-extract" is placed the amount of fat extracted by the wet method from the contents of the thimble after thirty hours' extraction by the Folin-Wentworth method. It will be seen that the combined fatty residues of the extract and the re-extract are equal to the result obtained ~~from~~ a like amount of specimen by the wet method.

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It might be said here that both methods give identical results on specimens to which infusorial earth has not been added. It would seem that the earth causes a small portion of the fats to enter into a colloidal combination which ether will not break up. In the wet method this combination is destroyed in an ether-water-alcohol menstrum.

TABLE II.

NO.	WEIGHT OF SPECIMEN	WEIGHT OF TOTAL FATS	FATTY ACID TITRE IN GRAMS
1	0.5	0.0275	0.0113
2	0.5	0.0274	0.0099
3	0.5	0.0276	0.0114

TABLE III.

NO.	WEIGHT OF TOTAL FATS IN RE-EXTRACT	FATTY ACID TITRE IN GRAMS OF RE-EXTRACT
1	0.0040	0.0022
2	0.0033	0.0025
3	0.0040	0.0022

TABLE IV.

NO.	FOLIN-WENTWORTH EXTRACT PLUS RE-EXTRACT BY WET METHOD	PERCENTAGE OF TOTAL FATS
1	$0.0275 + 0.0040 = 0.0315$	6.30
2	$0.0274 + 0.0033 = 0.0307$	6.14
3	$0.0276 + 0.0040 = 0.0316$	6.32

Average of three determinations, 6.25 per cent.

Compare the average of four determinations in Table I with that of three determinations in Table IV.

If it is thought advisable by the worker to extract larger samples it is necessary only to increase the size of the extraction cylinder and increase the quantities of water, alcohol and ether proportionately.

I have extracted the entire twenty-four-hour excreta of an eight months' old infant in a 250-cc. cylinder. In this case the quantities of reagents used were two and one-half times the amount used in a 100-cc. cylinder.

# THE IVES REPLICA DIFFRACTION GRATING IN SPECTROSCOPIC ANALYSIS.

By GORDON J. SAXON<sup>1</sup>

(From the Pepper Laboratory of Clinical Medicine, University of Pennsylvania.)

(Received for publication, January 3, 1914.)

In the examination of fluids giving absorption spectra it has been found convenient to use the Ives replica diffraction grating. This little device is used extensively by physicists in determining wave lengths.

In the examination of dilute solutions by transmitted light it enables one readily to take advantage of the fact that prolongation of the column under examination is equivalent optically to concentration of the fluid, as is the case in colorimetric determinations. Fluids containing oxyhemoglobin in such high dilution that it is impossible to get an absorption spectrum through a column 0.3 meter long, may display very distinct bands when a column a meter long is used.

I have demonstrated the absorption bands of oxyhemoglobin in a solution containing one drop of blood in 2 liters of water, simply by examining it through great depths of the fluid.

I have found it convenient to have three troughs of varying lengths, one-third meter, two-thirds meter and one meter respectively, the cross sections of which give a horizontal dimension of 5 mm. and a perpendicular dimension of 20 mm.<sup>2</sup> They are made by flattening glass tubing and cutting or grinding off the top. Parallel plane plates of glass are cemented on the ends. The best cement to use, I have discovered, is dislysin, obtained from bile since it withstands the action of acids, alkalies, ether, alcohol or chloroform. It is used hot and the surfaces to be cemented should first be submerged in boiling water.

<sup>1</sup>Woodward Fellow in Physiological Chemistry.

<sup>2</sup>The troughs used were made by Dolbey and Company, Philadelphia.

Figure 1 shows the arrangement of grating  $G$ , slit for transmission of light  $S$ , trough  $MN$ , collecting lens  $L$ , and source of illumination  $F$ . The best illumination is obtained from a Welsbach mantle; electricity may well be used however, especially if inflammable solutions are to be examined. The focal length of the lens is 15 to 20 cm. The source of light is placed at a distance from the lens equal to its focal length, thus producing parallel rays which pass through the glass plate  $N$  and through the solution. The rays emerge at  $M$  and strike a screen having an oblong slit about 0.5 mm. wide at  $S$ . The screen may be constructed from a thin piece of sheet copper or tin mounted on a stand by means of a clamp. This would permit up and down adjustment. The distance from the trough to the slit should be as small as possible but the difference from the slit to the diffraction grating  $G$  is arbi-

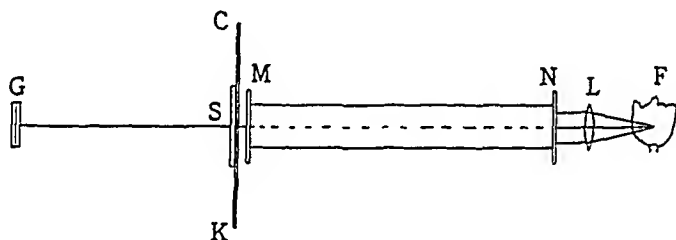


FIG. 1.

trary. It is, however, advisable to make the distance  $S-G$  at least 1 meter if measurements are desired.

The grating is transparent. If the eye is placed behind it the direct image of the slit  $S$ , can be seen. On either side of this direct image will appear spectra of light, the whole spectrum being visible at the same time. If rays of light from other sources should be seen in looking at the spectra, they may be cut off by use of a cardboard screen  $CK$ . This should extend 30–40 cm. either side of the slit. It may stand on the table in a perpendicular position and be made to support itself against the stand holding the slit screen.

The absorption spectrum does not consist of sharp lines missing from the continuous spectrum of the flame, but whole regions of the spectrum are absorbed. It is obvious that not only a color of

one wave length is absorbed in this region but many adjacent colors. If therefore it is desired to state between which wave lengths the absorption is to be found it may be easily determined. The width of the bands will vary with the concentration of the solution and length of the column through which the light has passed. The edges of the bands are not very sharply defined but they are much more easily identified than with an instrument permitting a view of only a small part of the spectrum.

Comparison of the absorption spectrum can easily be made with the direct or unabsorbed spectrum of the source. If the upper rays from the lens pass above the solution and the slit be made long enough to allow them to pass through, the observer can see

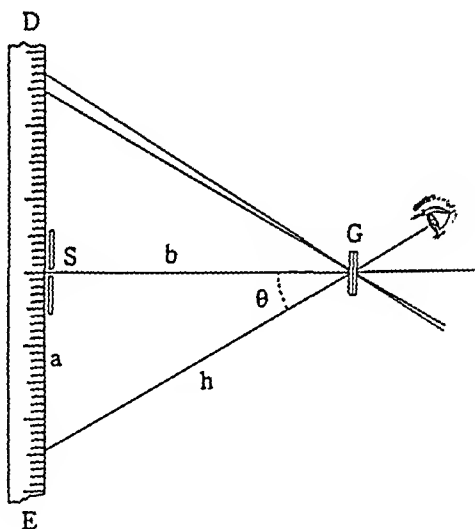


FIG. 2.

both these spectra at the same time. This renders the absorption spectrum more striking by comparison.

If it is desired to measure the wave lengths of the absorption bands a meter stick may be placed along *S*, as in figure 2. In looking at the spectrum with the eye the position of the band can be noted on the meter scale and the distance, *a*, determined. It is well to obtain this position on both sides of *S*, and take the aver-

It is well known that the antiketogenetic action of glucose is not due to the mere presence of glucose in the system, but to its oxidizibility and utilizability. From this we may justly conclude that the antiketogenetic effect of glucose is brought about either by the glucose molecule while undergoing oxidation, or by products that arise in its intermediary metabolism.

It was also of great moment to determine the rôle of the various radicals in a compound with reference to antiketogenesis. For example, in a molecule of glucose we find four secondary and one primary alcohol radicals and one aldehyde radical. The question then is this: Is it not possible that certain of these radicals are responsible for the antiketogenetic effects? In that case, other substances possessing these certain radicals may also be possessed of antiketogenetic powers. Also, by eliminating certain radicals and substituting others in the molecule it may be worked out to what extent each radical influences the pharmacological action of the substance. Proceeding in this way, we thought light might be thrown on the chemical prerequisites of antiketogenesis.

*Effect of d-gluconic acid on acidosis.*  $\text{COOH}-(\text{CHOH})_4-\text{CH}_2\text{OH}$

In experiment I a female dog was phlorhizinized and the nitrogen, glucose, acetone, aceto-acetic acid and  $\beta$ -hydroxybutyric acid eliminations were studied.<sup>2</sup>

EXPERIMENT I. *Twenty-four-hour periods.*

DATE Jan. 1912	PERIOD	WEIGHT	NITROGEN	GLUCOSE	D:N	ACETONE AND ACETO- ACETIC ACID	$\beta$ -HYDROXY- BUTYRIC ACID	REMARKS
23	II	10.16	13.03	45.06	3.46	0.276	0.94	{ 20.0 gms. of calcium gluconate given per os dissolved in 200 cc. of water.
24	III	9.86	10.49	37.67	3.59	0.342	1.26	
25	IV		9.54	35.74	3.73	0.360	1.48	
25	V	8.90	9.12	32.05	3.51	0.390	1.98	

<sup>2</sup> For details of experiments see previous communications, Ringer and Frankel: this *Journal*, xvi, p. 563, 1914.

In period III 20 grams of calcium gluconate, dissolved in 200 cc. of water, were given *per os*. As is seen from the table, calcium gluconate does not possess the power of reducing acidosis.

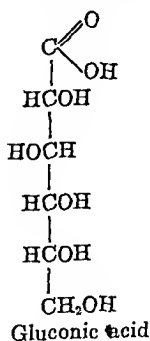
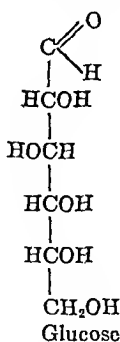
In experiment II period V, 25 grams of calcium gluconate were given *per os* with similarly negative results.

EXPERIMENT II. Twenty-four-hour periods.

DATE Feb. 1912	PERIOD	WEIGHT	NITROGEN	GLUCOSE	D:N	ACETONE AND ACETO- ACETIC ACID	$\beta$ -HYDROXY- BUTYRIC ACID	REMARKS
1	III	14.56	11.46	41.73	3.64	0.249	0.870	
2	IV	13.91	10.08	36.53	3.62	0.432	1.97	
3	V	13.21	9.43	31.17	3.30	0.500	2.18	{ 25.0 gms. of calcium gluconate given as above.
4	VI	12.49	9.56	31.13	3.25	0.472	1.88	

There is no question that gluconic acid is burnt in the animal body. This has been proven by the work of Salkowski,<sup>3</sup> Baumgarten<sup>4</sup> and P. Mayer.<sup>5</sup>

When one considers the close similarity that obtains between the structure of the gluconic acid molecule and that of glucose, and notes that the only difference between the two is the fact that the



<sup>3</sup> Salkowski: *Zeitschr. f. physiol. chem.*, xxvii, p. 507, 1899.

<sup>4</sup> Baumgarten: *Centralbl. f. Physiol.*, xx, p. 24, 1906; *Zeitschr. f. exp. Path. u. Ther.*, ii, p. 53, 1905.

<sup>5</sup> P. Mayer: *Zeitschr. f. klin. Med.*, xlvii, p. 68, 1902.

glucose molecule possesses an aldehyde radical, which in gluconic acid is oxidized to a carboxyl, *one feels justified in assuming that with the aldehyde radical rests the difference in the pharmacological action of these two substances.*

This assumption gains great weight when we realize in what a complexity of unions the glucose molecule enters because of the combining power of the aldehyde radical. There are a great many compounds, especially alcohols and ketones, which when introduced into the animal body, enter into chemical union with the aldehyde radical of the glucose molecule, thus giving rise to glucuronates. O. Neubauer<sup>6</sup> made a very systematic study of all the alcohols and found that the secondary alcohols possess the greatest power for this union. When ketones are given to animals, they are reduced to the stage of secondary alcohols before they enter into similar unions.

In a previous communication<sup>7</sup> we showed that acetaldehyde, when administered subcutaneously to diabetic dogs, causes a very marked depression in the acidosis, with a concomitant rise in the glucose elimination. The amount of extra glucose was much greater than could be accounted for by a complete conversion of the acetaldehyde into glucose. It is therefore evident that acetaldehyde possesses the power of causing certain substances which are ordinarily non-glucogenetic to become glucogenetic.

It was suggested that the antiketogenetic and the glucogenetic effects of acetaldehyde were causally related and that, in all probability, acetaldehyde united with  $\beta$ -hydroxybutyric acid, giving rise to  $\beta$ -methyl levulinic acid, which after undergoing demethylation, may give rise to levulinic acid, which in its metabolism, may give rise to propionic or pyruvic acid as intermediary bodies, which finally go over into glucose.

At present we are inclined to suggest the following possible intermediary reactions.

I. A condensation of acetaldehyde with  $\beta$ -hydroxybutyric acid forming  $\beta$ -methyl- $\beta,\gamma$ -dihydroxyvalerianic acid.

II. This after undergoing demethylation may give rise to  $\beta,\gamma$ -dihydroxyvalerianic acid.

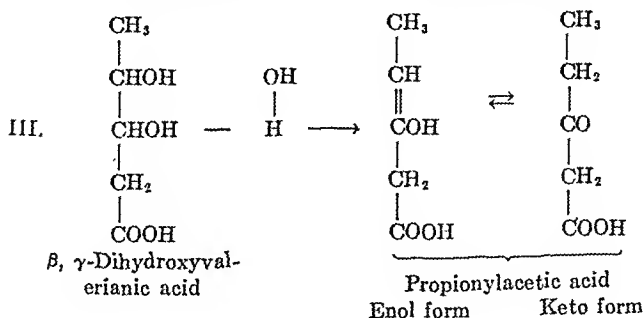
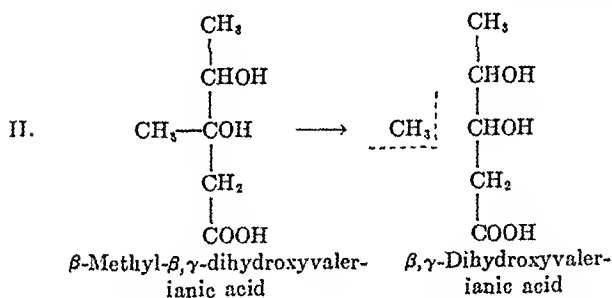
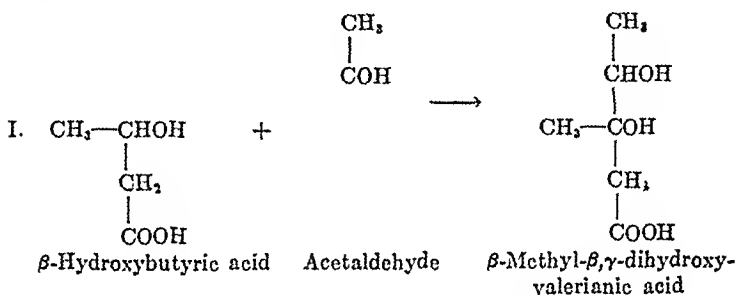
<sup>6</sup> O. Neubauer: *Arch. f. exp. Path. u. Pharm.*, xlii, p. 133, 1901.

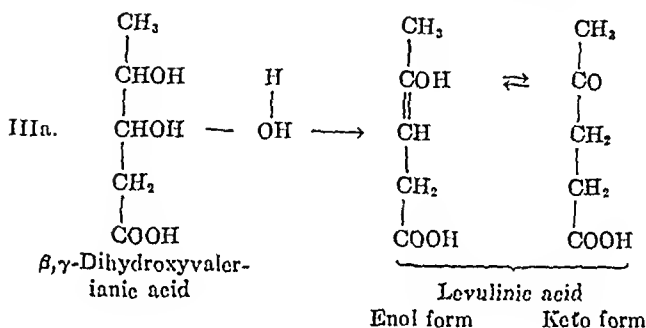
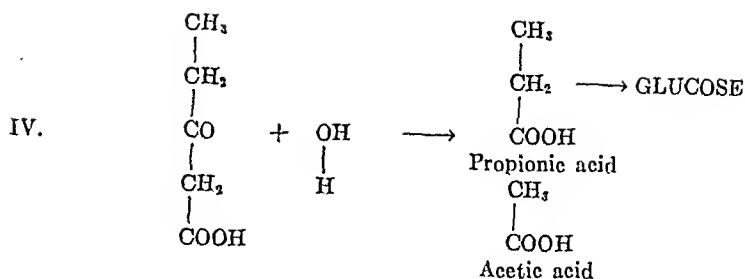
<sup>7</sup> Ringer and Frankel: *this Journal*, xvi, p. 563, 1914.

III. On losing a molecule of water it may give rise to propionylacetic acid.

IV. This substance on further oxidation, may give rise to propionic acid which goes over into glucose.

IIIa. The  $\beta, \gamma$ -dihydroxyvalerianic acid may also give rise to a certain amount of levulinic acid. But we no longer believe that the main reaction proceeds along this path. For levulinic acid was not found to be as strong a glucogenetic substance as was originally thought.



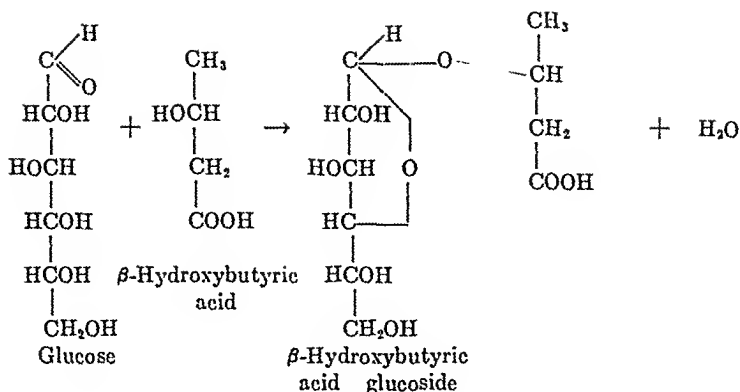


It must be realized that these reactions are at present entirely hypothetical. They do, however, serve to show how an aceton-genetic substance may be diverted from its ordinary path of catabolism to another path more favorable to the animal organism, namely glucogenetic.

It seems to us that there is sufficient justification, both experimental and from analogy, for assuming that the antiketogenetic action of glucose proceeds along similar lines. As it has been definitely established that secondary alcohols possess great power of combining with the glucose molecule through the aldehyde radical, giving rise to glucosides and glucuronates,<sup>8</sup> and as it has also been established that these bodies undergo oxidation in the animal body,<sup>9</sup> it seems reasonable to assume that the glucose molecule may combine with  $\beta$ -hydroxybutyric acid in the following way:

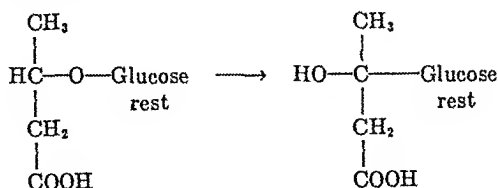
<sup>8</sup> Emil Fischer: *Ber. d. d. chem. Gesellsch.*, xxvi, p. 2400, 1893.

<sup>9</sup> P. Mayer: *loc. cit.*

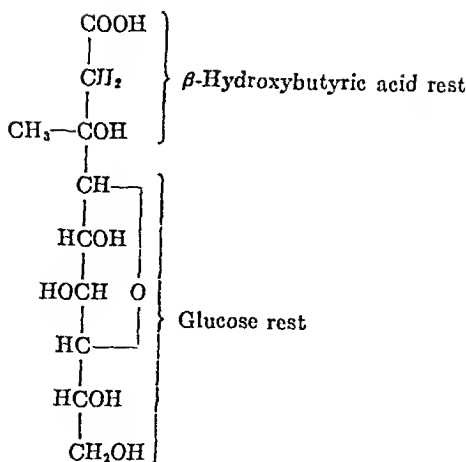


*In this combination the  $\beta$ -hydroxybutyric acid rest cannot possibly become oxidized to a ketone stage, i.e., to aceto-acetic acid, because that oxidation can be accomplished only by a removal of two atoms of hydrogen of the  $\beta$ -carbon and this compound possesses there only one available hydrogen. It is evident therefore, since oxidation does take place, that the point of attack must be other than the  $\beta$ -carbon.*

There is another possibility to be borne in mind, which may be operative in rendering the  $\beta$ -carbon of the  $\beta$ -hydroxybutyric acid fraction of the glucoside immune to further oxidative processes, and that is the possibility of this substance undergoing an intramolecular isomeric change, a transference of the glucose attachment from the oxygen to the  $\beta$ -carbon.



The straight-chain four-carbon compound may thus be converted into an iso-compound



which, after undergoing demethylation,<sup>10</sup> could give rise to a three carbon rest, which cannot possibly give rise to acetone bodies.

We may therefore conceive of the rôle of glucose in the normal individual in preventing acidosis to be such as to deviate the  $\beta$ -hydroxybutyric acid from its ordinary course of oxidation, by combining with it and thereby changing its structural configuration so as to give rise to non-acetone genetic products.

Reasoning on the basis of the theory presented above, two factors seem to be necessary for the prevention of acidosis:

I. The presence of an abundance of carbohydrates in the diet to combine with all of the  $\beta$ -hydroxybutyric acid as it is produced in the intermediary metabolism of the fatty acids, leucine, tyrosine, etc.

II. The ability of the individual to accomplish the "glucoside union" with  $\beta$ -hydroxybutyric acid.

The first prerequisite is a well established fact and was recognized by Rosenfeld,<sup>11</sup> Hirschfeld,<sup>12</sup> Geelmuyden,<sup>13</sup> Satta,<sup>14</sup> and a

<sup>10</sup> Baer and Blum: *Arch. f. exp. Path. u. Pharm.*, lv, p. 89, 1906; Embden, Solomon and Schmidt: *Hofmeister's Beiträge*, viii, p. 129; Ringer, Frankel and Jonas: *this Journal*, xiv, p. 525, 1913.

<sup>11</sup> Rosenfeld: *Deutsch. med. Wochenschr.*, xi, p. 683, 1885.

<sup>12</sup> Hirschfeld: *Zeitschr. f. klin. Med.*, xxviii, p. 176, 1895; Hirschfeld was really the first to give the current interpretation to the value of the carbohydrates in preventing acidosis. In 1885 it was still believed that the ace-

great many clinical observers. They found that in all conditions of nutrition of normal individuals the reduction of carbohydrates in the diet below a certain minimum produces acidosis. This acidosis is generally proportional to the amount of fat burned. If our theory is correct, an amount of glucose that *just* suffices to prevent acidosis in a normal individual on a fat-protein diet should fail to inhibit acidosis when the fat catabolism is increased by hard work. Whether this quantitative relationship does exist between the amount of glucose in the intermediary metabolism and the amount of fatty acids catabolized, will be the subject of our studies in the near future.

The second prerequisite is the more important one. It is well known that carbohydrates do not act antiketogenetically merely by being present in the circulation, for in the diabetic we may find the highest degree of acidosis associated with marked hyperglucæmia. It has always been stated that glucose must "burn in the body" before it can affect the oxidation of  $\beta$ -hydroxybutyric acid, and that the diabetic, having lost the power to burn glucose, consequently loses the power to oxidize  $\beta$ -hydroxybutyric acid and aceto-acetic acid.

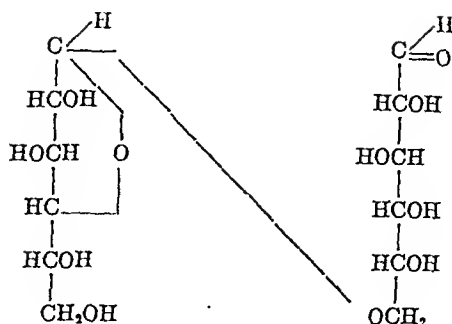
In our theory we assume that glucose acts antiketogenetically by forming a glucoside compound with  $\beta$ -hydroxybutyric acid. It seems reasonable to further assume, that *since glucose and  $\beta$ -hydroxybutyric acid circulate in abundance in the blood of the diabetic, the immediate cause of acidosis may be due to the failure of that individual to accomplish this glucoside union.* As  $\beta$ -hydroxybutyric acid arises in metabolism, it circulates in the blood as such, and becomes partly oxidized to aceto-acetic acid, which, in turn, is converted into acetone, all three substances being finally eliminated in the urine.

We believe the failure of the diabetic to form glycogen may be explained on exactly the same basis. In the formation of glycogen a glucose molecule attaches itself to another glucose molecule, forming a glucose-glucoside or maltose.

tone bodies came from protein, and Rosenfeld's explanation then was, that the carbohydrates depressed acidosis, by depressing the protein catabolism.

<sup>13</sup> Geelmuyden: *Zeitschr. f. physiol. Chem.*, xxiii, p. 470, 1892.

<sup>14</sup> Satta: *Hofmeister's Beiträge*, vi, p. 1, 1906.



This glucose-glucoside with its free aldehyde radical attaches itself to a similar molecule giving rise to a carbohydrate with four glucose components. This reaction goes on, constantly increasing the size of the molecule, until the glycogen stage is reached. The chemical characteristic of all these unions is the same, namely "glucoside formation."

No one any longer questions the correctness of the observation that the formation of glycogen is very much restricted in the diabetic. The restriction in the glycogen formation tends to run parallel, in a general way, with the severity of the diabetes and the degree of acidosis.

We consider the correlation of these phenomena on the basis of pure coincidence unjustified, and it seems reasonable to us, and in harmony with all the facts that acidosis, *i.e.*, the failure to burn  $\beta$ -hydroxybutyric acid, and the failure to form glycogen with the resultant hyperglucæmia, are genetically related because of the failure on the part of the individual to accomplish the "glucoside union."

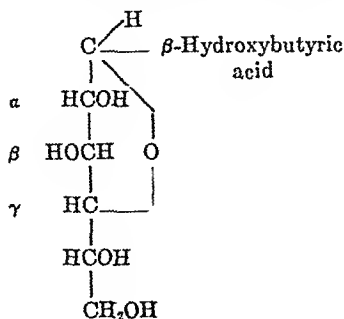
The observations of Levene and Meyer<sup>15</sup> showing that the action of pancreatic and muscle extracts on glucose is to cause a condensation of glucose to maltose, *i.e.*, the accomplishment of the glucoside union, is of great interest in this connection and lends support to our theory. This discovery, which gives a true interpretation of Cohnheim's old observation that pancreatic and muscle extracts have the power of causing a reduction in the reducing power of a sugar solution, is very significant and will be referred to again.

There is another very important problem in the physiology of diabetes which we believe may be brought into harmony with the

<sup>15</sup> Levene and Meyer: this *Journal*, ix, p. 98, 1911.

preceding. Since the influence of carbohydrates on acidosis became known twenty years ago, it has been taught that "carbohydrates cause the complete combustion of the fatty acids." The question we wish to raise here is this: Is it not possible that at the same time that the carbohydrates influence the combustion of the fatty acids, the fatty acids also influence the combustion of glucose, and that the effect is really a mutual one? Diabetes is the only condition in which severe forms of acidosis are seen, and although this acidosis is independent of the extent of the glucosuria, it is entirely dependent upon the amount of glucose the body is capable of burning. Improvement in the power of glucose combustion results in greater combustion of the fatty acids, while a decrease in the power of glucose combustion results in a decrease in the combustion of the fatty acids. May we not reverse this statement and ask if it may not also be true that improvement in the power of combustion of the fatty acids results in an improvement in the power of glucose combustion, and that a decrease in the power of combustion of the fatty acids results in a decrease in the power of sugar combustion? In other words, is it not possible that these two phenomena are not cause and effect, but run parallel to each other, governed and regulated by the same force, representing two aspects of the same reaction?

In our theory we propose that the glucose influences the combustion of the fatty acids by forming glucoside combinations with them. Fischer<sup>16</sup> has demonstrated that in every formation of glucosides, an ether-like combination is formed between the carbon of the aldehyde radical and the  $\gamma$ -carbon of the glucose molecule:



<sup>16</sup> Emil Fischer: *loc. cit.*

Is it not possible then, that this may act as an entering wedge for oxidative processes which finally cause a disruption of the glucose rest into two three-carbon compounds, and that a break in the glucose molecule does not take place unless this internal combination, which takes place only in case of glucoside formation, has been previously established? This would explain the parallelism between the failure in glucose combustion and acidosis.

The influence of the pancreas on carbohydrate metabolism is, in a general way, pretty well understood. It stimulates the formation of glycogen in the liver and muscles, it causes the combustion of sugar, and thus indirectly the complete combustion of the fatty acids, etc. From the work of Levene and Meyer<sup>17</sup> it is evident that the pancreas does not possess the power of glucolysis, but that its activity centers around its ability to accomplish the glucoside formation, which reaction is reversible and regulated by the laws of mass action.

In the presence of a pure and concentrated solution of glucose, the reaction proceeds only between glucose and glucose. In the presence of glucose and  $\beta$ -hydroxybutyric acid or lactic acid, it is possible that three glucosides are formed: glucose glucoside (maltose),  $\beta$ -hydroxybutyric acid glucoside, lactic acid glucoside.<sup>18</sup> Factors of relative concentration probably determine the character of the glucoside formed.

Taking all of the aforesaid into consideration we are inclined to believe that *the failure to accomplish the glucoside union is at the bottom of all the chemical disturbances in diabetic individuals. The failure to form glycogen, with the consequent hyperglucæmia, the failure to burn glucose, and the disturbance in the combustion of the lower fatty acids, can all be explained on the basis of this theory.*

This failure may come on gradually, affecting first the maltose and glycogen formation, which becomes manifested by lowered sugar tolerance, hyperglucæmia and alimentary glucosuria. As

<sup>17</sup> Levene and Meyer: *loc. cit.*

<sup>18</sup> Lactic acid being an hydroxy-acid may be conceived of entering into combination with glucose in a way similar to  $\beta$ -hydroxybutyric acid, forming a lactic acid glucoside, which may also influence the glucose combustion. This will explain the oxidation of large quantities of glucose when on a diet so rich in carbohydrates as to exclude the possibility of combustion of fatty acids from fat.

the disease progresses the glycogen formation becomes more and more interfered with, until we have permanent glucosuria.

As long as enough glucoside-genetic function remains to cause the conjugation of all the hydroxy fatty acids as they arise in the intermediary metabolism of the fatty acids and of protein, so long will acidosis not come into evidence. When, however, this begins to fail, acidosis appears, and the diabetic enters upon the "moderately severe" stage when even the sugar that originates from the metabolism of protein is not completely utilized. When the glucoside-genetic power of the individual fails completely, we then deal with the most severe type of diabetes, which is associated with complete failure in sugar combustion, giving a D:N ratio of about 3.6, when on a fat-protein diet and a degree of acidosis which corresponds to almost the theoretical amount of  $\beta$ -hydroxybutyric acid that can arise from the fat and protein metabolism.

We have thus shown how the different phenomena in diabetes can be harmonized and explained on the basis of the individual's loss of power to conjugate glucose in the formation of glucosides. Many of the questions that have been raised in this thesis cannot yet be considered fully answered. Further investigations along different paths of this problem are in progress, and we hope they may serve to make many a dubious point clear. This theory represents the line of reasoning which guides us in our continued researches in the chemotherapy of diabetes.



# THE ACTION OF VARIOUS ANAESTHETICS IN SUPPRESSING CELL-DIVISION IN SEA-URCHIN EGGS.

By RALPH S. LILLIE.

(From the Marine Biological Laboratory, Woods Hole, and the Biological Laboratory, Clark University.)

(Received for publication, January 9, 1914.)

Cell-division is initiated in unfertilized eggs by various external changes of condition (exposure to mechanical shock, heat, the electric current, various chemical substances) which are largely identical with the "stimuli" that call forth the characteristic reactions in irritable tissues like muscle and nerve. The unfertilized egg-cell must thus be regarded as an irritable element which responds by a characteristic and special type of activity to the action of any adequate stimulus, provided this is not in itself injurious. After the initiation of cell-division the process continues automatically in a rhythm which though slow is often very regular. The rate of rhythm shows a functional dependence on external conditions similar to that shown by various other physiological rhythms, such as the rhythm of contraction in heart-muscle or cilia, or of the generation of impulses in automatic nerve ganglia. All of these processes exhibit similar temperature coefficients, a similar dependence on the reaction and electrolyte content of the media, and a similar susceptibility to temporary retardation or arrest in the presence of various lipid-solvent substances. Such resemblances suggest strongly that the physiological conditions controlling the rate of rhythm are of the same essential nature in all of these different cases, and various indications point to the semi-permeable surface films or plasma membranes, common to all of these irritable elements, as the structures most directly concerned in the regulation of this rhythm. Some process in the nature of an alternate dissolution and reformation of the protoplasmic surface films, or of some similar reversible change of composition or aggregation-state—analogueous to that conditioning the rhythm in the mercury-

hydrogen peroxide catalysis studied by Bredig—seems to form the basis of this regular periodicity. That a surface process forms the primary condition of this phenomenon is directly implied by the more general features of the stimulation process. The work of Nernst and his successors has shown that changes in the electrical polarization of the plasma membranes form the primary or critical event in the electrical stimulation of irritable tissues; the same is probably true of other forms of stimulation also—since in a given tissue the different stimuli elicit the same response, which is always preceded by an electromotor change.<sup>1</sup> It is clear from the case of heart muscle that cells exhibiting an automatic rhythm differ from other irritable cells, as regards the essential conditions of their activity, only in the automaticity and regular recurrence of the normal stimulating condition—whatever the nature of this may be. Hence we must assume that a periodic variation in the electrical polarization of the membranes of such cells forms an essential underlying condition of the rhythm which their activity shows. The external evidence of this rhythmical surface process is the rhythmical electrical variation or action current; this is readily demonstrated in rhythmical muscle and nerve cells; and there is some experimental indication that a similar electrical variation accompanies cell-division in egg-cells,<sup>2</sup> although further evidence is needed on this point.

The case of cells undergoing a regular series of cell-divisions belongs thus in the category of automatic rhythmical processes, and the effect of anaesthetics in suppressing cleavage forms simply a special case of the general phenomenon of anaesthesia. It seems, therefore, highly probable that the physiological change which renders a cell temporarily incapable of division is of the same nature as that which temporarily deprives a nerve or muscle of

<sup>1</sup> Cf. my discussion of the general relation of membranes to stimulation in *Amer. Journ. of Physiol.*, xxviii, p. 197, 1911. It is interesting that the rhythmical mercury-peroxide catalysis of Bredig may also under certain conditions be initiated by the passage of an electric current, and that the product of the intensity of the effective current into the square root of its minimum duration appears to approach constant value, as Nernst's law of electrical stimulation requires. Cf. Bredig and Wilke: *Biochem. Zeitschr.*, xi, p. 67, 1908.

<sup>2</sup> Cf. the experiments of I. H. Hyde: *Amer. Journ. of Physiol.*, xii, p. 241, 1904.

irritability. The examination of the effects produced by anaesthetics on dividing egg-cells thus forms one means—hitherto little employed—of investigating the general nature and conditions of anaesthetic action. In many respects egg-cells form highly favorable objects for the experimental study of this problem. Any changes in the mechanical or other properties of the surface films, as shown, *e.g.*, by changes in their physical consistency or by an altered resistance to cytolytic agents, may be studied far more readily in such cells than in muscle or nerve. There is the further advantage that the individual cells can be examined separately; also that the properties and behavior of such cells as *Arbacia* eggs are remarkably constant.

During the past summer at Woods Hole I have investigated the effects produced by anaesthetics on the two separate processes: (1) normal cell-division in fertilized *Arbacia* eggs, and (2) the artificial initiation of cell-division in unfertilized eggs through the action of pure isotonic solutions of neutral salts (0.55 M KCNS and NaI). The existence of certain physiological antagonisms between salts and anaesthetics<sup>3</sup> suggested the possibility that the cleavage-initiating action of salt solutions might be inhibited by anaesthetics, and this was found to be the case. The basis of both of these inhibitions appears to be a modification in the properties of the surface films. The prevention of a characteristic surface process, the formation of the fertilization membranes, forms indeed an especially clear and unequivocal indication of the truth of the general point of view advocated in this and preceding papers<sup>4</sup> regarding the physico-chemical basis of anaesthetic action. These experiments will be described more fully in a later paper. The present paper will deal more particularly with the inhibiting action of anaesthetics on normal cleavage.

In a recent series of papers<sup>5</sup> I have brought forward evidence that the plasma membranes of irritable elements undergo during anaesthesia definite changes in their properties, becoming temporarily more resistant to the permeability-increasing action of pure solutions of sodium chloride and other salts. Since increased

<sup>3</sup> Cf. my recent papers in *Amer. Journ. of Physiol.*, xxix, p. 372, 1912; xxx, p. 1, 1912; xxxi, p. 255, 1913.

<sup>4</sup> *Loc. cit.* Cf. also *Science*, N.S., xxxvii, p. 959, 1913.

<sup>5</sup> *Loc. cit.*

resistance to this action is associated with a condition of decreased or abolished irritability, *i.e.*, of increased resistance to the action of stimulating agencies, and since stimulation appears to require a temporary increase in the general permeability of the plasma membranes,<sup>6</sup> the inference seems justified that this change in the properties of the limiting membranes is the essential condition that determines the loss of irritability during anaesthesia.<sup>7</sup> Accordingly I have put forward the hypothesis that the physico-chemical basis of anaesthetic action is a temporary change in the properties of the plasma membranes, of such a kind that these structures become relatively resistant to, or incapable of, the rapid and reversible variations of permeability and of electrical polarization that form the normal primary physiological condition of the excitation process.<sup>8</sup> By preventing changes in the membranes, anaesthetics may also lessen or prevent injury due to disintegrative changes in these structures; hence an anti-cytolytic action is frequently associated with the anti-stimulating action. This effect is simply another consequence of the increased stability or resistance to change imparted by the anaesthetic to the membranes. Since cell-division appears also to be accompanied by periodic variations of permeability and of electrical polarization,<sup>9</sup> we must infer, consistently with our general hypothesis, that the anaesthetic interferes with or suppresses cell-division by rendering the plasma membranes of the dividing cells temporarily incapable of undergoing these changes, just as it suppresses stimulation by inhibiting the membrane changes associated with this process. The plasma membranes of egg-cells appear to be similar in their physico-chemical constitution to those of muscle or

<sup>6</sup> Cf. my papers cited above.

<sup>7</sup> Loss of irritability is only one of the demonstrable effects associated with anaesthesia. Increased resistance to the action of cytolytic agents is another. Both appear to depend on the same condition, namely, a temporary alteration in the properties of the plasma membranes. The decrease in oxidations usually accompanying anaesthesia is probably also due to this condition.

<sup>8</sup> That is, stimulation involves a series of events interconnected in a definite sequence, beginning with the excitatory external change. The first change in the living system according to the present conception is a surface change of the nature indicated.

<sup>9</sup> Cf. my paper in *Amer. Journ. of Physiol.*, xxvi, p. 126, 1910.

other irritable cells. The presence of lipoids is indicated by the fact that lipid solvents may show anti-cytolytic action, as well as by the readiness with which lipid-soluble substances gain entrance to the cell-interior as compared with chemically similar but lipid-insoluble substances.<sup>10</sup> This similarity in the constitution of the membranes is in all probability the essential basis of the similarity in the action of lipid-solvent anaesthetics on such outwardly dissimilar processes as muscular contraction and cell-division.

Comparatively few experiments have been made on the anaesthetization of cell-division. Claude Bernard's experiments on the influence of ether in arresting the growth of seedlings<sup>11</sup> belong among the earliest observations of this kind, since cessation of growth implies the arrest of cell-division. Of systematic experiments with dividing cells those of Fühner<sup>12</sup> are the most extensive. He experimented with eggs of the sea-urchins, *Psammechinus* and *Strongylocentrotus*, and determined the concentrations of a number of anaesthetics, particularly alcohols, which had equal effects in producing a definite retardation of development. He found for the series of monohydric aliphatic alcohols that each member of the group was from three to four times as effective (for equimolecular concentrations) as its immediate predecessor. He found the same rule to hold—as Overton had found before him<sup>13</sup>—for neuromuscular anaesthesia in a large number of animals belonging to various groups.<sup>14</sup> He interprets his results as favoring the Overton-Meyer theory of narcosis, rather than that of Traube, according to whom the increase of narcotic action in the series of alcohols runs parallel with the increase in the surface activity<sup>15</sup> of the compounds rather than with the increase in their lipid-water partition coefficients. According to Traube's rule the rate of

<sup>10</sup> The contrast between the rapid entrance of  $\text{NH}_4\text{OH}$  and the lack of entrance of  $\text{NaOH}$  or  $\text{KOH}$  is the best illustration of this. *Arbacia* eggs exhibit this contrast in a typical manner.

<sup>11</sup> C. Bernard: *Leçons sur les phénomènes de la vie communs aux animaux et végétaux*, Paris, 1878. Chapter vii, pp. 259, et seq.

<sup>12</sup> H. Fühner: *Arch. f. exp. Path. u. Pharm.*, lii, p. 69, 1904.

<sup>13</sup> Overton: *Studien über die Narkose*, Jena, 1901, p. 100.

<sup>14</sup> Fühner: *Zeitschr. f. Biol.*, lvii, p. 465, 1912.

<sup>15</sup> That is, effect in lowering surface-tension at phase boundaries in aqueous solution.

increase of narcotic action in successive members of the homologous series would be slower than that observed by Fühner. Fühner did not determine the concentrations required for the complete and reversible arrest of cell-division, and no comparative studies of this kind appear hitherto to have been made.

#### EXPERIMENTAL.

The action of a considerable number of anaesthetics on cell-division in the fertilized eggs of *Arbacia punctulata* was investigated in the following manner. After fertilization with spermatozoa the eggs were left in normal sea-water until the first cleavage was completed (from one to one and a quarter hours after insemination at the summer temperature of the water, 20-23°). While in the two-cell stage and before any external signs of the second cleavage were visible, a considerable number of eggs were transferred with pipettes to small Erlenmeyer flasks; after the eggs had settled the sea-water was removed as far as possible, and replaced by sea-water containing known concentrations of the anaesthetics under investigation. After the lapse of a period known to be sufficient to permit of several successive divisions in normal sea-water—varying from two to five hours in different experiments—the eggs were examined, and the changes undergone, if any, were noted; they were then replaced in normal sea-water, washed free of anaesthetic by several changes of sea-water, and left over night. The proportion proceeding with development to a free-swimming larval stage was determined the next morning.

These experiments have shown that the different anaesthetics vary widely in their ability to arrest cell-division completely, for a considerable space of time (two to five hours), without causing permanent and fatal injury to the eggs. The same effect may be produced by depriving the eggs of oxygen or by potassium cyanide. Cyanide causes complete arrest of cleavage in concentrations so low as  $\frac{M}{8000}$ , for periods of five or six hours, without appreciably interfering with subsequent development.

Of the anaesthetics examined, chloral hydrate and urethanes have proved the most effective; the higher alcohols from propyl up are also favorable, though somewhat less so. Ethyl ether and chloroform are quickly fatal in solutions of the strength necessary

to arrest cleavage completely; the same is true of chlorotone, paraldehyde, and to an even greater degree of nitromethane, acetone, and ethyl nitrate. Certain anaesthetics are surprisingly ineffective, *e.g.*, acetanilide, phenyl urea, and chloralose; 0.5 per cent solutions of these substances caused no evident retardation of cleavage and had no toxic effect during exposures of two and a half hours;<sup>16</sup> these solutions cause typical neuromuscular anaesthesia in *Arenicola* larvae and with tadpoles they are still more effective (Overton). In general the concentrations required for complete arrest of cell-division in *Arbacia* eggs are relatively high as compared with those required for neuromuscular anaesthesia in marine and other animals. This appears from a comparison with the data given by Fühner, Overton, Vernon<sup>17</sup> and others. In its resistance to anaesthetization the cell-division process resembles ciliary movement. No definite grounds for the specific favorability of certain anaesthetics and the unfavorability of others can at present be assigned. Similar differences are observed for neuromuscular anaesthesia in different animals, and in the same animal at different ages. Divergences, qualitative and quantitative, in the lipid content of the tissues, and hence of the plasma membranes, form most probably the physico-chemical basis for these physiological differences. The fact that not all anaesthetics are capable of arresting cell-division completely for a considerable length of time without injury to the eggs corresponds to the fact that only a small proportion of the many known narcotic compounds can be used to advantage in medicine for practical anaesthesia. Accessory irreversible effects seem readily produced by these substances as a class, and the difference between the anaesthetic and the cytolytic concentrations is small in many cases. Such compounds are unfavorable for these purposes.

Observations were made last summer with the following compounds: ethyl ether, chloroform, chloral hydrate, chlorotone, methyl, ethyl, and phenyl urethanes, ethyl, propyl, isopropyl,

<sup>16</sup> One per cent morphine sulphate also had no perceptible effect on cleavage for the first few hours, though eventually it proved fatal; in 0.5 per cent solutions the great majority of eggs developed to the blastula stage. Morphine is similarly inactive with tadpoles according to Overton (*Narkose*, p. 170).

<sup>17</sup> Vernon: *Journ. of Physiol.*, xliii, p. 325, 1911; *ibid.*, xlvii, p. 15, 1913.

*n*-butyl, *i*-amyl and capryl alcohols, phenyl urea, chloralose, acetanilide, nitro-methane, acetonitrile, ethyl nitrate, paraldehyde. Observations were also made with potassium cyanide for comparison.<sup>18</sup>

The following is a summary account of the observations made with the different compounds.

*Ether.* Solutions of ether of a concentration sufficient to prevent cleavage completely are rapidly fatal to *Arbacia* eggs. In solutions of 0.8 and 0.6 vol. per cent the great majority of eggs were found to undergo cytolysis within three hours. In the 0.4 vol. per cent solution cleavage proceeds though slowly; but three hours' exposure to this solution proved fatal to the majority of eggs, only 30 to 40 per cent reaching the blastula stage.

*Chloroform.* This compound is also highly toxic to *Arbacia* eggs. An exposure of about three hours to one-sixth and one-eighth saturated solutions in sea-water proved fatal to all eggs. A few (less than 1 per cent) survived exposure to one-twelfth saturated solution for two and three-quarters hours, and formed blastulae.

*Chloral hydrate.* This anaesthetic is the most effective of any that I have thus far tried. In appropriate concentrations (*ca.* 0.1 per cent) it suppresses cleavage for hours without causing evident injury. Five series of experiments (twenty-one single experiments) with concentrations ranging from 0.3 to 0.02 per cent were performed with this compound last summer. In all cases cleavage was completely prevented by concentrations of 0.1 per cent and above. A majority of eggs were found to form blastulae after three hours' exposure to 0.3 and 0.2 per cent solutions; but an exposure of four hours to the 0.2 per cent solution proved fatal to all eggs. Most (*ca.* 70 per cent) formed blastulae after five and a half hours in the 0.1 per cent solution. In somewhat weaker solutions (0.08 and 0.06 per cent) a small proportion of eggs

<sup>18</sup> Squibb's ether, chloroform and ethyl alcohol were used, Kahlbaum's chloral hydrate, and Parke, Davis & Co. chlorotone; the remaining anaesthetics were from Merck. The amyl alcohol used in these experiments, and also in my former experiments with *Arenicola* larvae and starfish eggs, was the iso-alcohol,  $(CH_3)_2CH \cdot CH_2-CH_2OH$ , and not the normal alcohol—as was stated by an inadvertence in the papers referred to (*loc. cit.* 1913, and *Journ. of Exp. Zoölogy*, xv, p. 23, 1913).

TABLE I.  
*Chloral hydrate.*

The eggs were placed in the following solutions of chloral hydrate in sea-water about one hour and fifteen minutes after insemination. At that time most were in the two-cell stage; a minority were still uncleaved. Eggs were returned to sea-water at two intervals after placing in the solutions, (a) 2 hours, 45 minutes; (b) 5 hours, 30 minutes.

CONCENTRATION OF CHLORAL HYDRATE	CONDITION OF EGGS AFTER ca. 3 HOURS IN SOLUTIONS	PROPORTION OF EGGS FORMING BLAS- TULAE AFTER EXPOSURE TO SOLUTIONS FOR (a) 2½ HOURS; (b) 5½ HOURS
<i>per cent</i>		
0.16	All are in two-cell stage or uncleaved	(a) ca. 70-80% form blastulae. (b) ca. 25 %; most eggs dead.
0.14	All in two-cell or uncleaved	(a) ca. 70 % form blastulae.
0.12	All in two-cell or uncleaved	(a) majority form blastulae. (b) large majority form blas- tulae: ca. 80-90 %.
0.01	All in two-cell or uncleaved	(a) majority form blastulae (70% or more). (b) majority form blastulae (ca. 70 %).
0.08	Almost all in two-cell or un- cleaved	(a) Less favorable than pre- ceding: ca. 50 % form blas- tulae. (b) Most eggs dead; ca. 20- 30 % form blastulae.
0.06	Most remain in two-cell but a considerable number have cleaved again	(a) ca. 50 % form blastulae. (b) a small proportion form blastulae: ca. 5 %.

usually cleave; in the 0.04 per cent solution the majority cleave slowly, while in 0.02 per cent there is little apparent retardation, at least during the first two or three hours.

It is remarkable that the eggs withstand prolonged exposure (five to six hours) to solutions of a concentration not quite sufficient entirely to prevent cleavage less readily than to slightly stronger solutions that completely inhibit this process. This is illustrated by the experiments summarized in Table I, which show an optimum of survival in 0.12 and 0.1 per cent solutions; below which there is a marked decline in the proportion of developing eggs. Two other similar series of experiments showed the same phenomenon. Apparently the injurious action of chloral hydrate is greater when the cleavage process, though slowed, is still able

partly to continue, than during its complete suppression. The susceptibility to injury by this substance thus appears greater during even slight activity than during complete rest. This observation recalls the difference observed by Loeb between resting (unfertilized) eggs and dividing eggs in their relative susceptibility to injury by pure sodium chloride and other toxic solutions.<sup>19</sup> During complete anaesthesia the cell appears to be protected against the injurious action of the anaesthetizing substance itself. Possibly its penetration into the egg interior is then more difficult. There are various indications that during cleavage the surface permeability undergoes increase.<sup>20</sup> Chloral hydrate has a relatively low lipid-water partition coefficient, so that its entrance is probably readier at times of increased permeability. Some indication of a similar phenomenon was seen with ethyl urethane but not with the other anaesthetics.

*Chloretone.* Chloretone is much less effective than chloral hydrate in suppressing cleavage. Even one-third saturated solutions which proved fatal to all eggs within two and a half hours, permitted irregular cleavage in a large proportion of eggs. Solutions of half this concentration (one-sixth saturated) produce complete neuromuscular anaesthesia in *Arcincola* larvae.<sup>21</sup> A striking feature of the action of chloretone is a rapid liberation of pigment from the eggs. This occurs in concentrations which entirely fail to prevent cleavage ( $\frac{1}{10}$  and  $\frac{1}{12}$  saturated), and in the weaker solutions is accompanied by little if any immediate toxic action, so that it resembles a secretion process rather than an indication of cytolysis. A similar though less pronounced effect of this kind is produced by capryl alcohol. Four series of experiments were performed with chloretone using the concentrations  $\frac{1}{3}$ ,  $\frac{1}{4}$ ,  $\frac{1}{5}$ ,  $\frac{1}{6}$ ,  $\frac{1}{8}$ ,  $\frac{1}{10}$ ,  $\frac{1}{12}$  saturated in sea-water.<sup>22</sup>

<sup>19</sup> J. Loeb: *Biochem. Zeitschr.*, ii, p. 81, 1907.

<sup>20</sup> Cf. my paper in *Amer. Journ. of Physiol.* already cited, xxvi, p. 126. I have observed under certain conditions (eggs together with an excess of spermatozoa under a cover glass) an exit of pigment from the eggs during the appearance of the cleavage furrow: other eggs in the same preparation not yet cleaved showed no such change. This observation was first made in the physiology class at Woods Hole by R. A. Spaeth.

<sup>21</sup> *Amer. Journ. of Physiol.*, xxix, p. 387, 1912.

<sup>22</sup> The saturated solution of this compound is approximately 1 per cent (ca. 0.045 M).

TABLE 11.  
*Chlorethane.*

CONCENTRATION OF CHLORETHANE	CONDITION OF EGGS AFTER REMAINING 3 HOURS IN SOLUTIONS*	PROPORTION OF EGGS FORMING BLASTULAE AFTER EXPOSURE TO SOLUTIONS FOR (a) 2½ HOURS; (b) 5½ HOURS
(1) $\frac{1}{2}$ saturated	Many remain in 2-cell stage, but most have cleaved irregularly	(a) None form blastulae; all are dead next day. (b) The same.
(2) $\frac{1}{4}$ saturated	Fewer 2-cell stages than in Exp. 1; irregular cleavages more advanced	(a) ca. 50 % form blastulae. (b) Nearly all die early; ca. 5 % form blastulae.
(3) $\frac{1}{8}$ saturated	Majority have undergone irregular cleavage; a fair proportion remain in 2-cell stage	(a) ca. 70-80 % form blastulae. (b) ca. 40 % form blastulae, mostly irregular.
(4) $\frac{1}{16}$ saturated	Most have formed irregular groups of 8-16 cells. A few remain in 2-cell stage	(a) Great majority (ca. 90 %) form blastulae. (b) ca. 50 % form blastulae.

\* In all of these solutions the eggs rapidly lose pigment.

Table II summarizes the results of a typical series of experiments. The procedure was the same as in the series of Table I.

*Urethanes.* Methyl, ethyl and phenyl urethanes all cause typical reversible arrest of cleavage. For methyl urethane the effective concentrations lie between 2.5 and 2 per cent. The 3 per cent solution causes cytolysis within two hours, and in the 1.5 per cent solution cleavage proceeds, though slowly. With ethyl urethane five series of experiments were performed. The effective concentrations were found to lie between 1.75 and 1.5 per cent. Only 5 per cent of the eggs survived exposure to the 2 per cent solution for two and a quarter hours, and in the 1.25 per cent solutions cleavage continued slowly. Observations were made with solutions of the following percentage concentrations: 2, 1.75, 1.6, 1.5, 1.25, 1, 0.8, 0.75, 0.6, 0.4, 0.3, 0.2; the last three solutions had little or no perceptible effect within the time occupied by the experiment.

Table III summarizes the results of a typical series of experiments with ethyl urethane. The eggs were placed in the solution at one and a quarter hours after fertilization; nearly all were then in the two-cell stage.

TABLE III.

*Ethyl urethane.*

CONCENTRATION OF ETHYL URETHANE	CONDITION OF EGGS AFTER 2½ HOURS IN SOLUTIONS	PROPORTION OF EGGS FORMING BLASTULAE AFTER EXPOSURES OF (a) 2½ HOURS; (b) 5½ HOURS
<i>per cent</i>		
(1) 1.75	All are in 2-cell stage or un-cleaved	(a) <i>ca.</i> 90 % form blastulae. (b) <i>ca.</i> 90 % form blastulae.
(2) 1.5	All are in 2-cell stage or un-cleaved	(a) <i>ca.</i> 90 % form blastulae. (b) <i>ca.</i> 90 % form blastulae.
(3) 1.25	Most remain uncleaved or in 2-cell, but a few have cleaved again	(a) <i>ca.</i> 90 % form blastulae. (b) <i>ca.</i> 90 % form blastulae.
(4) 1.0	Large proportion have cleaved to irregular 4-cell groups	(a) <i>ca.</i> 90 % form blastulae. (b) <i>ca.</i> 80 % form blastulae; somewhat more eggs die than in preceding experiments.
(5) 0.75	Most eggs are in irregular 4- and 8-cell groups	(a) Nearly all form blastulae ( <i>ca.</i> 90 %). (b) Nearly all form blastulae.

TABLE IV.

*Phenyl urethane.*

CONCENTRATION OF PHENYL URETHANE	CONDITION OF EGGS AFTER 2½ HOURS IN SOLUTIONS	PROPORTION FORMING BLASTULAE AFTER EXPOSURE TO SOLUTIONS FOR 2 HOURS
<i>per cent</i>		
(1) 0.1	Eggs remain in 2-cell stage or uncleaved	Majority break down; <i>ca.</i> 25-35 % form blastulae.
(2) 0.08	Cleavage is arrested completely as in Exp. 1	A considerable proportion break down; <i>ca.</i> 50-60 % form blastulae.
(3) 0.06	Arrest of cleavage is not quite complete. A few eggs in 4-cell stage	Most form blastulae.
(4) 0.04	About 50 % remain in 2-cell stage or uncleaved; the rest have undergone irregular cleavage or change of form	Most form blastulae, but these are less active than normal (few swimming at the surface).

Phenyl urethane is also a highly effective narcotic with *Arbacia* eggs. Table IV summarizes four observations with this compound. The anaesthetizing concentrations lie between 0.08 and 0.06 per cent; two hours' exposure to the 0.1 per cent solution is fatal to most eggs, while 0.04 per cent allows irregular cleavage in a large proportion. A second similar series with the concentrations 0.1, 0.075, 0.05 and 0.025 per cent gave the same result.

*Alcohols.* A large number of experiments were performed with the following monohydric alcohols: ethyl, propyl, isopropyl, *n*-butyl, *i*-amyl, capryl. In appropriate concentrations all of these suppress cleavage for periods of two to five hours without preventing subsequent development. Propyl, butyl and amyl alcohols are more favorable than ethyl or capryl, *i.e.*, the suppression of cleavage in anaesthetic concentrations is more complete and is attended with less injury to the eggs; the favorability increases in the order propyl, butyl, amyl. Ethyl and capryl alcohols show a higher specific toxicity than the others. Methyl alcohol was not tried.

The following tables summarize the results of several typical experiments with these alcohols. The procedure was the same as in the foregoing experiments. The eggs were introduced into the

TABLE V.  
*Ethyl alcohol.*

CONCENTRATION OF ETHYL ALCOHOL	CONDITION OF EGGS AFTER 3 HOURS IN SOLUTIONS	PROPORTION FORMING BLASTULAE AFTER EXPOSURE TO SOLUTIONS FOR (a) 2½ HOURS; (b) 5½ HOURS
<i>vol. per cent</i>		
(1) 6	Most eggs remain in 2-cell stage. Cytolysis well marked	No development.
(2) 5	About one-third remain in 2-cell stage; rest are irregularly cleaved or fragmented	(a) Most dead next day; <i>ca.</i> 10 % form blastulae. (b) blastulae fewer: <i>ca.</i> 5 %.
(3) 4	Cleavage is slowed, but most eggs have cleaved irregularly	(a) Most form blastulae: <i>ca.</i> 80-90 %. (b) Nearly all are dead next day; <i>ca.</i> 5 % form blastulae.
(4) 3	Cleavage has proceeded slowly. Eggs are in irregular cell groups of 4 to 16 cells	(a) Most form blastulae: 80-90 %. (b) Blastulae numerous but fewer: <i>ca.</i> 50-60 %.

solution of the alcohols in sea-water while in the two-cell stage, and the proportion forming blastulae on return to sea-water after definite intervals was approximately determined.

*Ethyl alcohol.* Solutions of 6 vol. per cent and above are quickly fatal. Solutions sufficiently strong to suppress cleavage completely are rapidly injurious. In 5 vol. per cent solutions the anaesthetization is incomplete; but even this solution is fatal to most eggs within three hours.

*Propyl alcohol.* Solutions of 2 vol. per cent concentration arrest cleavage completely in most eggs, and are only gradually toxic. 2.5 per cent solutions are fatal to all eggs within three to four hours. Four series of experiments were performed with concentrations ranging from 4 vol. per cent to 1.25 vol. per cent. The following series of four experiments is typical.

TABLE VI.  
*n-Propyl alcohol.*

CONCENTRATION OF <i>n</i> -PROPYL ALCOHOL	CONDITION OF EGGS AFTER 3 HOURS IN SOLUTIONS	PROPORTION FORMING BLASTULAE AFTER EXPOSURE TO SOLUTIONS FOR (a) 2½ HOURS; (b) 5½ HOURS
vol. per cent		
(1) 2	Most eggs remain in 2-cell stage. A fair number have formed irregular 4- to 8-cell groups	(a) Most form blastulae: ca. 80 %. (b) Fewer blastulae: ca. 30-40 %.
(2) 1.75	Most remain in 2-cell stage. Proportion of cleaved eggs is higher than in Exp. 1	(a) ca. 90 % form blastulae. (b) ca. 40 % form blastulae.
(3) 1.5	Most eggs have cleaved; but many remain in 2-cell stage	(a) 80-90 % form blastulae. (b) 50 % form blastulae.
(4) 1.25	Most have cleaved to irregular 8-16 cell groups	(a) ca. 90 % form blastulae. (b) ca. 50-60% form blastulae.

*Isopropyl alcohol.* All observers agree that this alcohol has a less pronounced anaesthetic action, in equimolecular concentrations, than the normal alcohol. This is true also for cell-division. Even in the 3 vol. per cent solution cleavage was found to continue, though slowly, in most eggs. After three hours in the 2 vol. per cent solution most were found irregularly cleaved to groups of 8-16 cells.

*n-Butyl alcohol.* Cleavage is almost completely suppressed in solutions of 1 vol. per cent to 0.8 vol. per cent. Exposure for

TABLE VII.  
*n*-Butyl alcohol.

CONCENTRATION OF <i>n</i> -BUTYL ALCOHOL	CONDITION OF EGGS AFTER 3 HOURS IN SOLUTIONS	PROPORTION FORMING BLASTULAE AFTER EXPOSURE TO SOLUTIONS FOR (a) 2½ HOURS; (b) 5½ HOURS
<i>vol. per cent</i>		
(1) 0.8	Most eggs are in 2-cell stage; a few 4- or 8-cell groups	(a) ca. 80 % form blastulae. (b) ca. 25-30 % form blastulae.
(2) 0.7	Most eggs have cleaved once or twice; majority form ir- regular 8-cell or 16-cell groups	(a) ca. 90 % form blastulae. (b) ca. 50 % form blastulae.
(3) 0.6	Few remain in 2-cell stage. Most are irregular 8-16 cell groups	(a) ca. 90 % form blastulae. (b) Less than 50 % form blas- tulae.
(4) 0.5	Cleavage is retarded less than in Exp. 3; most eggs form groups of ca. 32 cells. A few 4- and 8-cell groups	(a) ca. 90 % forms blastulae. (b) ca. 40-50 % form blastu- lae.

TABLE VIII.  
*i*-Amyl alcohol.

CONCENTRATION OF <i>i</i> -AMYL ALCOHOL	CONDITION OF EGGS AFTER 2½ HRS. IN SOLUTIONS	PROPORTION FORMING BLASTULAE AFTER EXPOSURE TO SOLUTIONS FOR (a) 2½ HOURS; (b) 5½ HOURS
<i>vol. per cent</i>		
(1) 0.5	None show further cleavage. All are in 2-cell stage or un- cleaved	(a) Most eggs die; ca. 10-15 % form blastulae. (b) None form blastulae.
(2) 0.45	Nearly all remain in 2-cell stage or uncleaved. A few 4-cells.	(a) Most eggs form blastu- lae; ca. 65-75 %. (b) Most eggs die; ca. 30-40 % form blastulae.
(3) 0.4	Nearly all remain in 2-cell stage or uncleaved. A few 4-cells	(a) ca. 80-90 % form blastu- lae. (b) ca. 80-90 % form blastu- lae.
(4) 0.35	A large proportion have cleaved irregularly to 4- or 8-cell groups	(a) ca. 90 % form blastulae. (b) ca. 90 % form blastulae.
(5) 0.3	Most have cleaved slowly, forming irregular 4- and 8- cell groups	(a) ca. 90 % blastulae. (b) ca. 90 % blastulae.
(6) 0.25	Almost all are cleaved; most- ly irregular 8-cell groups	(a) ca. 90 % blastulae. (b) ca. 90 % blastulae.

four hours to solutions of 1.6, 1.4 and 1.2 vol. per cent killed all eggs; exposure for three and a half hours to the 1 vol. per cent solution was also fatal to the majority, but a good many survived and formed blastulae. A favorable anaesthetic concentration is 0.8 vol. per cent. There were four series of experiments with this alcohol. The results summarized in Table VII are typical for concentrations of 0.8 vol. per cent and lower.

*i-Amyl alcohol.* 0.45 to 0.4 vol. per cent is a favorable anaesthetic concentration; 0.5 vol. per cent is somewhat rapidly toxic. Five series of experiments were performed with this alcohol. The observations summarized in Table VIII are typical for the anaesthetizing range of concentrations.

*Capryl alcohol.* This alcohol even in sub-anaesthetic concentrations exhibits a relatively high specific toxicity. Solutions of 0.02 per cent and above kill all eggs in three hours or less; in solutions of 0.018 to 0.015 per cent a reversible anaesthetic action is seen if the exposure is relatively short (three hours or less). In weaker solutions cleavage continues, though retarded. In all the solutions examined from 0.05 per cent to 0.01 per cent the eggs lose pigment, though this effect is slight in concentrations lower than 0.015 per cent. Three series of experiments (14 single determinations) were performed with this alcohol. Table IX summarizes a series of observations with the anaesthetizing range of concentrations.

The results of experiments with several other anaesthetics may be briefly summarized as follows.

*Phenyl urea, acetanilide, chloralose.* Each of these three compounds was used in the four concentrations 0.5, 0.25, 0.125 and 0.06 per cent. All are alike ineffective in preventing cleavage, which showed no evident retardation in any solution during exposures of two and a half hours. On return to sea-water development proceeded normally.

*Nitromethane* ( $\text{CH}_3\text{NO}_2$ ). In solutions of 5, 4, and 3 vol. per cent no cleavage occurred and all eggs died within three hours. In the 2 vol. per cent solution cleavage was also completely arrested, but a few eggs (5 to 10 per cent) survived three hours' exposure and formed blastulae.

*Acetonitrile* ( $\text{CH}_3\text{CN}$ ). Exposure for three hours to 5, 4, 3 and 2 vol. per cent solutions killed all eggs. There were indications of irregular cleavage in the 2 vol. per cent solution.

TABLE IX.

*Capryl alcohol.*

CONCENTRATION OF CAPRYL ALCOHOL	CONDITION OF EGGS AFTER 2½ HRS. IN SOLUTIONS	PROPORTION FORMING BLASTULAE AFTER 2½ HRS. IN SOLUTIONS
<i>per cent</i>		
(1) 0.02	Most eggs are cytolized. No further cleavage. A few normal 2-cells.	Very few eggs ( <i>ca.</i> 1 %) form blastulae.
(2) 0.018	Most eggs are cytolized. A considerable number of normal 2-cell groups present.	<i>ca.</i> 20-30 % form blastulae.
(3) 0.016	Most in 2 cells; a small proportion show irregular cleavage (4 cells).	Most eggs die: <i>ca.</i> 35-45 % form blastulae.
(4) 0.014	Cleavage has gone on slowly; most have formed irregular 4-8-cell groups.	Most eggs form blastulae: <i>ca.</i> 70-80 %.
(5) 0.012	Almost all have cleaved irregularly to groups of 4 to 16 cells.	Most form blastulae: <i>ca.</i> 80 %.

*Ethyl nitrate* ( $C_2H_5NO_3$ ). Exposure for three hours to solutions of 1, 0.75, 0.5, and 0.25 vol. per cent killed all eggs. A few showed indications of having cleaved in the 0.25 per cent solution.

*Paraldehyde*. Solutions of 5, 4, 3 and 2 vol. per cent were tried. The first three solutions (three-hour exposures) caused cytolysis in all eggs; there was some cleavage in the 3 vol. per cent solution. In the 2 vol. per cent solution cleavage continued irregularly in many eggs and about 35-45 per cent formed blastulae on return to normal sea-water.

*Potassium cyanide*. Observations with this compound were made for comparison. Solutions of concentrations ranging from  $\frac{N}{500}$  to  $\frac{N}{8000}$  were found to prevent cleavage entirely for periods of four to five and a half hours without impairing to any appreciable degree the subsequent development in normal sea-water. In the  $\frac{N}{10000}$  KCN a few eggs cleaved once within the three hours of exposure but the majority remained unaltered. Cyanide thus prevents cell-division entirely at concentrations far below those at which any anaesthetic so far studied produces an appreciable effect. This fact is in itself strongly suggestive of a fundamental difference in the respective modes of action of cyanide and of anaesthetics.

*Conclusion and summary.*

On comparing the results of the above experiments with those of my recent similar experiments with *Arenicola* larvae a close correspondence is seen between the concentrations of a given anaesthetic required to arrest cell-division in *Arbaeia* eggs and those inhibiting muscular contractions in the larvae. This is especially true of the series of alcohols; the urethanes, ether, chlorotone, and chloral hydrate show similar but somewhat less close correspondence. Most of the other compounds used with *Arenicola* larvae (*e.g.*, the esters) are too injurious to *Arbaeia* eggs for satisfactory employment in experiments requiring prolonged anaesthetization. The parallelism in the case of those compounds whose toxic action is slight is so close as to leave no doubt of the essential identity of the physiological conditions underlying the inhibitory action in the two cases. The following table gives for a considerable number of compounds the concentrations that just suffice to prevent cleavage in *Arbaeia* eggs or muscular contractions in *Arenicola*. In most cases the approximate upper and lower limits of the anaesthetizing range of concentrations are given. To facilitate comparison with other similar data the concentrations are expressed in both percentage and molecular terms.

The experiments with *Arenicola* larvae have shown that the anaesthetic effect is correlated with a modification of the plasma membranes, these structures becoming more resistant to alteration by salt solutions as the tissue becomes less responsive to stimulation. That an increasingly resistant character is also imparted by anaesthetics to the plasma membranes of *Arbaeia* eggs is indicated by the results of my recent experiments on the influence of these substances in preventing the cytolytic action of salt solutions on unfertilized eggs.<sup>23</sup> Other experiments performed last summer<sup>24</sup> showing that the cleavage-initiating action of pure salt solutions (0.55 M KCNS and NaI) can also be prevented by anaesthetics point in the same direction. A modification of the surface layer or plasma membrane thus appears to form the determining condition of the anaesthetic action in dividing cells as well as in irritable elements like muscle cells.

<sup>23</sup> *Amer. Journ. of Physiol.*, xxx, p. 1, 1912.

<sup>24</sup> About to be published in the *Journ. of Exp. Zoölogy*.

TABLE X.  
Effective concentrations of anaesthetic.

ANAESTHETIC	ARABACIA EGGS	ARENICOLA LARVAE
		<i>per cent</i>
Methyl alcohol....		9-10 v. % (2.2-2.5 M)
Ethyl alcohol....	ca. 5 v. % (0.87 M)	6-7 v. % (1.0-1.2 M)
n-Propyl alcohol..	ca. 2 v. % (0.27 M)	2-3 v. % (0.27-0.4 M)
Iso-propyl alcohol	ca. 3 v. % (0.4 M)	ca. 3-4 v. % (0.4-0.5 M)
n-Butyl alcohol	ca. 0.8 v. % (0.086 M)	0.8-1.0 v. % (0.08-0.1 M)
i-Amyl alcohol	ca. 0.4 v. % (0.037 M)	0.25-0.4 v. % (0.024-0.037 M)
Capryl alcohol....	ca. 0.015 v. % (0.001 M)	ca. 0.015 v. % (0.001 M)
Methyl urethane..	2-2.5 % (0.27-0.33 M)	ca. 4 % (0.5 M)
Ethyl urethane....	1.5-1.75 % (0.15-0.19 M)	2-3 % (0.2-0.3 M)
Phenyl urethane..	0.08-0.1 % (0.005-0.006 M)	ca. 0.15 % (0.008 M)
Ethyl ether.....	0.5-0.6 v. % (0.05-0.06 M)	0.7-1 v. % (0.07-0.1 M)
Chloroform.....	ca. 0.06 % ( $\frac{1}{12}$ sat.) (0.005 M)	$\frac{1}{8}$ - $\frac{1}{2}$ saturated (0.007-0.01 M)
Chloral hydrate...	0.1-0.12 % (0.006-0.007 M)	0.1-0.2 % (0.006-0.012 M)
Chloretone.....	0.2-0.25 % (0.008-0.01 M)	0.16-0.25 % (0.007-0.01 M)
Nitromethane.....	ca. 2. v. % (0.42 M): unfavourable	ca. 2.5 v. % (0.5 M)
Acetonitrile.....	ca. 2. v. % (0.5 M): unfavourable	4-5 v. % (1-1.25 M)
Ethyl nitrate.....	ca. 0.25 v. % (0.025 M): unfavourable	0.35-0.5 v. % (0.035-0.05 M)
Paraldehyde.....	2-3 v. % (0.15-0.2 M): unfavourable	ca. 2.5 v. % (0.2 M)
Chloralose.....	ineffective in sat. sol. (ca. 0.6 %)	ca. 0.6 % (0.018 M): effect gradual
Acetanilide.....	ineffective in sat. sol. (ca. 0.5 %)	ca. 0.5 % (0.035 M)
Phenyl urea.....	ineffective in sat. sol. (ca. 0.5 %)	ca. 0.5 % (0.035 M)
Ethyl acetate.....		1-1.5 v. % (0.1-0.15 M)
Ethyl propionate.		0.4-0.5 v. % (0.035-0.042 M)
Ethyl butyrate....		0.13-0.25 v. % (0.01-0.02 M)
Ethyl valerianate		0.08-0.13 v. % (0.005-0.008 M)

## SUMMARY.

1. The concentrations of various anaesthetics required for complete and reversible arrest of cleavage in the fertilized egg of *Arbacia* were determined. The favorable anaesthetics (alcohols, urethanes, chloral hydrate) were found to produce this effect in concentrations closely similar to those causing typical neuromuscular anaesthesia in *Arenicola* larvae.

2. Neuromuscular anaesthesia in *Arenicola* larvae is plainly correlated with an altered condition of the plasma membranes of the irritable elements, involving increased resistance to the alterative or permeability-increasing action of salt solutions. The same condition seems to underlie the inhibitory action of anaesthetics on cleavage, since in those concentrations which arrest cell-division the anaesthetics protect unfertilized eggs against both the cytolytic and the cleavage-initiating action of salt solutions.

## CONCERNING THE ORGANIC PHOSPHORIC ACID OF COTTONSEED MEAL II.

FIFTH PAPER ON PHYTIN.

By R. J. ANDERSON.

(From the Chemical Laboratory of the New York Agricultural Experiment Station, Geneva, N. Y.)

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In the last report<sup>1</sup> from this laboratory we described certain crystalline barium salts of the organic phosphoric acid of cottonseed meal. We had also prepared and analyzed the free acid itself and described its properties and we also showed that on cleavage with dilute sulphuric acid in a sealed tube the substance gave inosite as one of the products of decomposition.

These crystalline barium salts and the free acid prepared from them gave results on analysis which differed slightly from corresponding compounds calculated on the usual formula for phytic acid, viz.,  $C_6H_{24}O_{27}P_6$ . The substance from cottonseed meal appeared to be an acid of the formula  $C_2H_6O_8P_2$  or  $C_6H_{18}O_{24}P_6$ . The barium salts agreed closely with this formula but the percentage of phosphorus in the free acid was found to be about 1 per cent lower than required.

The reactions of the aqueous solution of the free acid, however, were found to be identical in every respect with those given by phytic acid. From the results obtained we concluded that the organic phosphoric acid in cottonseed meal was very similar to phytic acid but we were unable to determine whether it was identical with this acid.

Prior to our publication, so far as we are aware, no definite organic phosphoric acid had ever been described as existing in cottonseed meal; no pure salts of this acid had been obtained nor had the free acid been prepared in pure form.

<sup>1</sup> This *Journal*, xiii, p. 311, 1912, and Technical Bulletin 25, N. Y. Agric. Exp. Station, 1912.

hydrogen and water and calculating to the free acid) the results agree very closely with the percentage composition calculated for inosite hexaphosphate.

In the isolation and purification of the barium salt we made use of our former method in preference to that proposed by Rather for the reason that we consider our method more simple and convenient. The essential difference in these methods of isolation being that we use barium hydroxide throughout, precipitating the substance with this reagent from dilute hydrochloric acid solutions. Rather used a modification of the method of Patten and Hart,<sup>7</sup> substituting the use of sodium hydroxide with ammonium hydroxide. The use of either sodium or ammonium hydroxide, which must be eliminated again, is not necessary, for barium hydroxide is equally efficient and by its use the introduction of other basic ions is avoided.

Since the present work substantiates our earlier results and since all the analytical data agree with inosite hexaphosphate,  $C_6H_{18}O_{24}P_6$ , or with salts of this acid we believe that the organic phosphoric acid in cottonseed meal must be represented by the formula either of inosite hexaphosphate,  $C_6H_{18}O_{24}P_6$ , or else some formula isomeric with this.

It may be noted that the percentage of phosphorus found on analyzing the free acid is somewhat low. In the analyses of the acids previously reported<sup>8</sup> the phosphorus was found to be from 1 to 1.8 per cent lower than required for inosite hexaphosphate. As will be shown in a later communication this is due to the fact that the free acid becomes largely hydrolyzed on drying.

#### EXPERIMENTAL PART.

##### *Isolation and purification of the barium salt.*

The cottonseed meal, 25 pounds, was digested over night in 0.2 per cent hydrochloric acid in porcelain percolators covered on the inside with a double layer of cheesecloth. It was then percolated using 0.2 per cent hydrochloric acid until about 20 liters of extract were obtained. The extract was of a dirty, dark

<sup>7</sup> *Amer. Chem. Journ.*, xxxi, p. 566, 1904.

<sup>8</sup> *Loc. cit.*

color and contained some suspended particles from which it was freed as much as possible by centrifugalizing the solution. A concentrated solution of 300 grams of barium chloride was then added and the precipitate allowed to settle. The precipitate was centrifugalized and finally brought upon a Buchner funnel and freed as far as possible from the mother-liquor. It was then digested in several liters of about 5 per cent hydrochloric acid until no further solution took place. The insoluble residue was removed by centrifugalizing and the still very dirty colored solution precipitated by adding barium hydroxide until the free acid was neutralized. The barium hydroxide was added slowly with constant shaking when the precipitate separated in crystalline form. It was then filtered and washed thoroughly in water and again dissolved in dilute hydrochloric acid, filtered and reprecipitated with barium hydroxide. These operations were repeated three times. The hydrochloric acid solution was then precipitated by gradually adding an equal volume of alcohol, when the substance again separated in crystalline form consisting of globular masses of microscopic needles. It was then precipitated a fourth time with barium hydroxide and after that two more times with alcohol. It was then filtered, washed free of chlorides with dilute alcohol and then in alcohol and ether and dried in vacuum over sulphuric acid. The product was then a nearly white, crystalline powder and it weighed 94 grams.

The dry substance was shaken up with about 1.5 liters of cold water, allowed to stand for several hours and then filtered and washed in water. The aqueous solution contained very little substance precipitable with alcohol and it was therefore discarded.

The washed precipitate was dissolved in dilute hydrochloric acid and precipitated a fifth time by the very gradual addition of barium hydroxide; after filtering and washing, this operation was repeated a sixth time. After again dissolving in dilute hydrochloric acid, nearly neutralizing the free acid with barium hydroxide and filtering, the substance was brought to crystallization by the gradual addition of an equal volume of alcohol. After standing for several hours the substance was filtered and washed in dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid. It was then a voluminous snow-white crystalline powder.

The dry substance was again dissolved in dilute hydrochloric acid, the free acid nearly neutralized with barium hydroxide and the solution filtered and allowed to stand over night. The substance soon began to crystallize. Under the microscope it appeared perfectly homogeneous and consisted as before of globular masses of microscopic needles. The substance was filtered, washed free of chlorides with water and then in alcohol and ether and dried in vacuum over sulphuric acid.

The dry, snow-white, crystalline powder weighed 69 grams.

Qualitative analysis failed to reveal any heavy metals other than barium, and from 0.5 gram of the salt no weighable residue of alkali was obtained. It gave no reaction with ammonium molybdate in nitric acid solution. It was free from sulphur and nitrogen.

It was analyzed after drying at  $105^{\circ}$  in vacuum over phosphorus pentoxide.

0.4641 gram substance gave 0.0556 gram  $H_2O$  and 0.1125 gram  $CO_2$ .

0.1982 gram substance gave 0.1333 gram  $BaSO_4$  and 0.1203 gram  $Mg_2P_2O_7$ .

Found: C = 6.61; H = 1.34; P = 16.91; Ba = 39.57 per cent.

For tri-barium inosite hexaphosphate,  $C_6H_{12}O_{24}P_6Ba_3 = 1066$ :

Calculated, C = 6.75; H = 1.12; P = 17.44; Ba = 38.65 per cent.

A portion of this salt was recrystallized as follows: 5 grams were dissolved in a small quantity of 5 per cent hydrochloric acid and the free acid nearly neutralized with barium hydroxide, the solution was then filtered and 2 grams of barium chloride dissolved in a little water added and the solution allowed to stand. The substance separated slowly in the usual crystalline form. After two days it was filtered, washed free of chlorides with water and again dissolved in the dilute hydrochloric acid, the solution filtered and alcohol added gradually until a slight cloudiness remained. After standing for twenty-four hours at room temperature the substance had crystallized in the usual form. It was filtered, washed free of chlorides in dilute alcohol and then in alcohol and ether, and dried in vacuum over sulphuric acid. The dilute nitric acid solution of the substance gave no reaction with ammonium molybdate. The snow-white crystalline powder was analyzed after drying at  $105^{\circ}$  in vacuum over phosphorus pentoxide.

0.3588 gram substance gave 0.0464 gram  $\text{H}_2\text{O}$  and 0.0867 gram  $\text{CO}_2$ .  
 0.1726 gram substance gave 0.1138 gram  $\text{BaSO}_4$  and 0.1058 gram  $\text{Mg}_2\text{P}_2\text{O}_7$ .  
 Found: C = 6.59; H = 1.44; P = 17.08; Ba = 38.79 per cent.

Another portion of the substance was recrystallized as follows: 2 grams were dissolved in a small amount of the dilute hydrochloric acid, barium hydroxide added with constant shaking until a faint permanent precipitate remained and the solution filtered. The filtrate was then heated to boiling and allowed to stand for a few minutes. As the temperature rose the solution began to turn cloudy and finally a heavy precipitate separated which appeared to be amorphous at first but it soon changed into the same crystal-form as previously described. This was filtered and washed free of chlorides in boiling water and then in alcohol and ether and allowed to dry in the air. The dry substance weighed 1.6 grams. The snow-white crystalline powder was free from inorganic phosphate. It was analyzed after drying at  $105^\circ$  in vacuum over phosphorus pentoxide.

0.4695 gram substance lost 0.0443 gram  $\text{H}_2\text{O}$ .  
 0.4252 gram substance gave 0.0423 gram  $\text{H}_2\text{O}$  and 0.0981 gram  $\text{CO}_2$ .  
 0.1238 gram substance gave 0.0885 gram  $\text{BaSO}_4$  and 0.0735 gram  $\text{Mg}_2\text{P}_2\text{O}_7$ .  
 Found: C = 6.29; H = 1.11; P = 16.54; Ba = 42.06;  $\text{H}_2\text{O}$  = 9.43 per cent.  
 For heptabarium inosite hexaphosphate,  $(\text{C}_6\text{H}_{11}\text{O}_{24}\text{P}_6)_2\text{Ba}_7 = 2267$ :  
 Calculated, C = 6.35; H = 0.97; P = 16.40; Ba = 42.39 per cent.  
 For  $14\text{H}_2\text{O}$ , calculated: 10.00 per cent.

Still another portion of the substance was recrystallized in the following manner: 2 grams were dissolved in the dilute hydrochloric acid and then nearly neutralized with barium hydroxide as before. The solution was filtered and 10 cc. of  $\frac{1}{2}$  barium chloride added and allowed to stand over night. The substance had been separated as a heavy crystalline powder of the same form as before except that the individual crystals were much larger. The crystals were filtered, washed free of chlorides with water and finally in alcohol and ether and allowed to dry in the air. It was analyzed after drying as above.

0.6430 gram substance lost 0.0745 gram  $\text{H}_2\text{O}$ .  
 0.5685 gram substance gave 0.0603 gram  $\text{H}_2\text{O}$  and 0.1258 gram  $\text{CO}_2$ .  
 0.2208 gram substance gave 0.1608 gram  $\text{BaSO}_4$  and 0.1252 gram  $\text{Mg}_2\text{P}_2\text{O}_7$ .

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Found: C = 6.03; H = 1.18; P = 15.80; Ba = 42.85; H<sub>2</sub>O = 11.58 per cent.

For heptabarium inosite hexaphosphate,  $(C_6H_{11}O_4P_6)_2Ba_7 = 2267$ :

Calculated, C = 6.35; H = 0.97; P = 16.40; Ba = 42.39 per cent.

For 16H<sub>2</sub>O, calculated: 11.27 per cent.

### *Preparation of the free acid.*

The acid was prepared in the usual way from 10 grams of the first crystalline barium salt. The aqueous solution finally obtained was concentrated in vacuum at 40°–45° to small bulk. It was then divided into three portions: one was dried in vacuum over sulphuric acid and then analyzed; the others were used for the preparation of the silver salts to be described later.

The dry acid was obtained as a practically colorless syrup. Its dilute aqueous solution gave no reaction with ammonium molybdate showing absence of inorganic phosphoric acid. Its reactions in other respects were identical with those previously described. For analysis it was dried first in vacuum over sulphuric acid at room temperature and finally in vacuum over phosphorus pentoxide at 105° when it turned quite dark in color:

0.3931 gram substance gave 0.1088 gram H<sub>2</sub>O and 0.1540 gram CO<sub>2</sub>.

0.1840 gram substance gave 0.1826 gram Mg<sub>2</sub>P<sub>2</sub>O<sub>7</sub>.

Found: C = 10.68; H = 3.09; P = 27.66 per cent.

For inosite hexaphosphate,  $C_6H_{13}O_4P_6 = 660$ :

Calculated, C = 10.90; H = 2.72; P = 28.18 per cent.

### *Preparation of the silver salt from the above acid.*

One portion of the free acid previously mentioned was dissolved in 100 cc. of water and the solution neutralized to litmus with ammonia. Silver nitrate solution was then added which caused a heavy, perfectly white, amorphous precipitate. This was filtered and carefully washed in water and dried in vacuum over sulphuric acid, the desiccator being kept in a dark place. After drying, the substance was a faintly cream colored powder which was very slightly sensitive to light. On moist litmus paper it showed a strong acid reaction. It was free from ammonia. For analysis it was dried at 105° in vacuum over phosphorus pentoxide.

On drying as above, not protected from light, the substance darkened somewhat in color.

0.3064 gram substance gave 0.0150 gram  $\text{H}_2\text{O}$  and 0.0446 gram  $\text{CO}_2$ .

0.1640 gram substance gave 0.1387 gram  $\text{AgCl}$  and 0.0596 gram  $\text{Mg}_2\text{P}_2\text{O}_7$ .

Found: C = 3.96; H = 0.54; P = 10.13; Ag = 63.65 per cent.

Deducting the above percentage of silver and allowing for an equivalent amount of hydrogen and water we obtain the following results:

Calculated: C = 10.74; H = 3.08; P = 27.45 per cent.

These percentages agree fairly closely with the composition calculated for inosite hexaphosphate, viz.:

C = 10.90; H = 2.72; P = 28.18 per cent.

To the remaining portion of the acid (about 5 cc.) 300 cc. of alcohol were added.<sup>9</sup> The solution remained perfectly clear. The alcohol was evaporated on the water bath and the residue taken up in 100 cc. of water in which it gave a slightly cloudy solution and which had a faint aromatic odor. The acid had possibly been esterified to a slight extent. It was filtered and neutralized to litmus with ammonia and precipitated with silver nitrate; the precipitate was filtered, washed in water and dried as before. The appearance of the precipitate was identical with that of the first one. On moist litmus paper it also showed a strong acid reaction and it was free from ammonia. For analysis it was dried as above.

0.4008 gram substance gave 0.0176 gram  $\text{H}_2\text{O}$  and 0.0600 gram  $\text{CO}_2$ .

0.1481 gram substance gave 0.1241 gram  $\text{AgCl}$  and 0.0548 gram  $\text{Mg}_2\text{P}_2\text{O}_7$ .

Found: C = 4.08; H = 0.49; P = 10.31; Ag = 63.06 per cent.

Calculated to the free acid as before the following percentages are obtained:

C = 10.88; H = 2.84; P = 27.52 per cent.

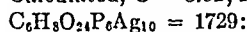
That the silver precipitates obtained under the above conditions do not represent homogeneous salts may be seen by comparing

<sup>9</sup> Mr. Rather found that his acid preparations gave a precipitate on addition of alcohol. This was no doubt due to the fact that inorganic bases had not been completely removed by his method of purification; hence an acid salt of the organic phosphoric acid was precipitated on the addition of alcohol. The acid prepared from our purified and recrystallized barium salts are completely soluble in alcohol.

the percentages found with the calculated composition of the following silver salts of inosite hexaphosphate:



Calculated, C = 3.92; H = 0.38; P = 10.13; Ag = 64.65 per cent.



Calculated, C = 4.16; H = 0.45; P = 10.75; Ag = 62.40 per cent.

Judging by the analytical results the amorphous silver precipitates appear to be mixtures of the above silver salts.

## CONCERNING PHYTIN IN OATS.

SIXTH PAPER ON PHYTIN.

By R. J. ANDERSON.

(From the Chemical Laboratory of the New York Agricultural Experiment Station, Geneva, N. Y.)

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In continuation of the investigation of the organic phosphoric acids of grains and feeding materials which has been carried out in this laboratory we have examined recently the compound existing in oats. This substance has already been studied by other investigators, notably by Hart and Tottingham<sup>1</sup> who came to the conclusion that oats contained phytin. The purpose of the present investigation was to determine whether the phytin in oats was identical with other phytin preparations obtained from other grains.

We have previously shown that cottonseed meal<sup>2</sup> contains an organic phosphoric acid which differs slightly in composition from that required for the phytic acid formula of Posternak, viz.:  $C_2H_8O_9P_2$ ; or according to Neuberg  $C_6H_{24}O_{27}P_6$ ; although so far as properties and reactions were concerned no differences could be observed. This acid from cottonseed meal had been isolated as a crystalline barium salt and since this salt did not show any change in composition on recrystallization we felt reasonably certain that it was a homogeneous substance. On the other hand we were unable to obtain any crystalline barium salts of the organic phosphoric acid of wheat bran.<sup>3</sup> Only amorphous salts were obtained, which differed entirely in composition from salts of phytic acid. It appeared of interest, therefore, to determine whether other grains contained organic phosphoric acids identical with those

<sup>1</sup> Wisconsin Agric. Exp. Station, Research Bulletin 9, 1910.

<sup>2</sup> This *Journal*, xiii, p. 311, 1912; N. Y. Agric. Exp. Station, Tech. Bulletin 25, 1912; and preceding article.

<sup>3</sup> This *Journal*, xii, p. 447, 1912; N. Y. Agric. Exp. Station, Tech. Bulletin 22, 1912.

previously described or if compounds of different composition were present.

In the present investigation the substance was isolated as a barium salt from 0.2 per cent hydrochloric acid extract of oats by precipitating with barium chloride. The substance was then repeatedly precipitated from dilute hydrochloric acid alternately with alcohol and with pure recrystallized barium hydroxide (Kahlbaum) until all bases other than barium were removed and until all the inorganic phosphate was eliminated.

Several preparations were made from different lots of oats. The substances showed absolutely no tendency to crystallize and they were all obtained as snow-white amorphous powders. On analysis these various preparations gave fairly concordant results but the composition differed considerably from that required for salts of phytic acid. The preparations were reprecipitated and subjected to various other treatments but were always recovered without showing any great variation in composition and it was therefore thought the substance was homogeneous.

However, it was found finally that these preparations, obtained by direct precipitation, were mixtures of barium salts; probably of two different organic phosphoric acids. Only one, however, has been isolated in pure form. By treating the above-mentioned amorphous barium salts with small quantities of cold water it was possible to effect a separation into two preparations having entirely different compositions. After the water-soluble portion had been removed the insoluble substance was found to crystallize readily in the same manner and in the same crystal-form as the barium salt obtained from the acid extracted from cottonseed meal, viz.: in round or globular masses of microscopic needles. Repeated recrystallizations did not alter the composition except as to the percentage of barium. When allowed to crystallize from dilute hydrochloric acid containing barium chloride a salt is obtained which contains from 40 to 42 per cent of barium but when it is brought to crystallization from dilute hydrochloric acid solutions by the addition of alcohol the salt contains about 38 per cent of barium. So far as one can judge by crystal-form, composition, properties and reactions the crystalline salts obtained from oats and cottonseed meal are identical.

The water-soluble substance referred to above could be obtained only as a snow-white amorphous powder. In composition it differed entirely from the crystalline product but very slightly from the compound isolated from wheat bran.<sup>4</sup> Owing to the amorphous nature of the substance, however, it is impossible to say at present whether it is a homogeneous body or merely a mixture of various compounds. We hope to study this matter more closely, particularly in comparison with the wheat-bran products which we propose to investigate further.

The composition of the crystalline barium salts obtained from oats and cottonseed meal does not agree with the usually accepted formula for phytic acid, viz.,  $C_6H_{24}O_{27}P_6$ . The analytical results of these preparations would indicate that they are salts of an acid of the formula  $C_2H_6O_8P_2$  or a multiple of it; probably  $C_6H_{18}O_{24}P_6$ . Such an acid would be isomeric or identical with inosite hexaphosphate which was suggested by Suzuki and Yoshimura<sup>5</sup> as the formula for phytic acid. We have always found, however, that the phosphorus in the free acid prepared from the above barium salts is always about 1 per cent lower than this formula requires. It is possible that this low percentage of phosphorus is due to partial hydrolysis in drying—which seems the more likely as the hydrogen is always found somewhat high. When the free acid is dried at a temperature of 100° or higher it turns perfectly black in color; even on drying at 60° or 78° in vacuum the color darkens perceptibly, which would indicate some decomposition. It will be shown later that hydrolysis actually does take place on drying and that a large percentage of the phosphorus in the dried preparations is present as inorganic phosphoric acid.

The analyses of the barium salts on the other hand agree very closely with the formula  $C_2H_4O_8P_2Ba$  or  $C_6H_{12}O_{24}P_6Ba_3$ . It will be shown also that the barium salts suffer but very slight hydrolysis on drying at a temperature of 105°. Evidently, therefore, it is safer to calculate the formula of the free acid from the barium salts rather than from analyses of the free acid itself.

<sup>4</sup> *Loc. cit.*

<sup>5</sup> *Coll. of Agric. Tokyo Bull.*, vii, p. 495.

## EXPERIMENTAL PART.

*Isolation of the substance.*

Whole ground oats, including grain and hull, were digested over night in 0.2 per cent hydrochloric acid in porcelain percolators covered on the inside with a double layer of cheesecloth. The next day the substance was percolated with the same strength hydrochloric acid until the extract gave no appreciable precipitate with barium chloride. The extract was then filtered through paper and precipitated by adding a concentrated solution of barium chloride in liberal excess. The precipitate, after settling, was filtered on a Büchner funnel and washed in 30 per cent alcohol. It was then dissolved in sufficient dilute hydrochloric acid, about 1 or 2 per cent, filtered and the filtrate precipitated with barium hydroxide solution. After settling, filtering and thoroughly washing with water the precipitate was again dissolved in the same strength hydrochloric acid as before, filtered and the filtrate precipitated by adding an equal volume of alcohol. After repeating the operations alternately a second time the substance was twice precipitated from dilute hydrochloric acid, same strength as above, with barium hydroxide (Kahlbaum) which had been recrystallized. It was then further precipitated three times from the same strength hydrochloric acid with alcohol. The final precipitate was filtered and washed free of chlorides with dilute alcohol and then in alcohol and ether and dried in vacuum over sulphuric acid.

The crude precipitate obtained by adding barium chloride to the acid extract of oats contains large quantities of impurities, inorganic phosphates, colored substances, etc., which during the above operations are gradually eliminated. The precipitates obtained at first do not dissolve completely in dilute hydrochloric acid. It is therefore necessary to repeatedly filter such solutions in order to free them from suspended insoluble matter. Finally, however, a product is obtained which is readily and completely soluble in the dilute hydrochloric acid, in which it gives a perfectly colorless solution.

When prepared as mentioned above, the dry substance is a snow-white, amorphous powder. It is very readily soluble in dilute hydrochloric and nitric acids, less so in acetic acid. It is

soluble to a considerable extent in cold water. On moist litmus paper it shows a strong acid reaction. Heated with hydrochloric acid and phloroglucine no appreciable color reaction developed. After boiling with dilute sulphuric acid for several minutes, filtering and neutralizing, it did not reduce Fehling's solution. It contained neither nitrogen nor sulphur and gave no reaction for chlorides. Dissolved in dilute nitric acid it gave no reaction with ammonium molybdate even after being kept at a temperature of  $65^{\circ}$  for some time and standing at room temperature for several days, showing that inorganic phosphates were absent. Bases, other than barium, could not be detected in 0.5 gram of the substance. Owing to loss in purification the yield is rather unsatisfactory. In one case 13 grams were obtained from 5 kgm. of oats; in another case 20 grams were obtained from 10 kgm. In all, four preparations were made, which gave a total of about 140 grams of the barium salt.

Much time was expended in an endeavor to obtain the substance in crystalline form, but as already mentioned it showed no tendency whatever to crystallize. The amorphous preparations were therefore analyzed after previous drying to constant weight at  $105^{\circ}$  in vacuum over phosphorus pentoxide. The following results were obtained:

- 1st preparation: C = 8.84; H = 1.67; P = 15.88; Ba = 36.72 per cent.  
2d preparation: C = 8.27; H = 1.47; P = 16.28; Ba = 37.26 per cent.  
3d preparation: C = 8.37; H = 1.60; P = 16.48; Ba = 36.79 per cent.  
4th preparation: C = 8.44; H = 1.61; P = 16.35; Ba = 36.61 per cent.

These results are fairly concordant but the composition differs considerably from that required for tri-barium phytate.

Calculated for  $C_6H_{18}O_{27}P_3Ba_3$ : C = 6.42; H = 1.60; P = 16.60; Ba = 36.78 per cent.

#### *Further purification of the barium salt.*

In order to determine whether the composition of the substance would change on further treatment the following experiment was tried. A portion of the first preparation was used. The barium was precipitated with slight excess of sulphuric acid, the barium sulphate filtered off and the filtrate precipitated with excess copper acetate. The copper salt was filtered and thorough-

washed in water and then suspended in water and decomposed with hydrogen sulphide. After removing the copper sulphide the filtrate was boiled to expel hydrogen sulphide and then precipitated with a solution of recrystallized barium hydroxide. Dilute hydrochloric acid was then added until the precipitate was just dissolved and the solution precipitated by adding an equal volume of alcohol. The precipitate was filtered, washed in dilute alcohol and then dissolved in 0.5 per cent hydrochloric acid and reprecipitated with alcohol. The precipitate was then filtered, washed in dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid. It was analyzed after drying at  $105^{\circ}$  in vacuum over phosphorus pentoxide.

Found: C = 8.23; H = 1.56; P = 16.19; Ba = 37.46 per cent.

In another case 6 grams of the second preparation were heated in a sealed tube with 45 cc. of 1.5 N sulphuric acid in the steam bath for twenty-four hours and then allowed to stand for four days at room temperature. After isolating in the same manner as above 3.7 grams were recovered. It was analyzed after drying as above.

Found: C = 8.42; H = 1.66; P = 16.17; Ba = 37.08 per cent.

These treatments apparently caused no change in composition.

The free acid was then prepared and analyzed. From the first preparation the acid was prepared in the usual way, *i.e.*, the barium was precipitated with slight excess of sulphuric acid, filtered and the filtrate precipitated with copper acetate. The copper salt was filtered, washed and decomposed with hydrogen sulphide, filtered and evaporated in vacuum at a temperature of  $40^{\circ}$ – $45^{\circ}$  and finally dried in vacuum over sulphuric acid. It was then obtained as a thick, practically colorless syrup. For analysis it was dried to constant weight over boiling chloroform in vacuum over phosphorus pentoxide. The color turned very slightly dark on drying in this way.

Found: C = 13.24; H = 3.26; P = 25.50 per cent.

The acid prepared from the repurified barium salt gave the following result on analysis after drying as above.

Found: C = 13.17; H = 3.39; P = 25.48 per cent.

The composition of the acid agrees with that required for the above barium salts and one might suppose from the close agreement of analytical results that the substance was homogeneous. It was found, however, that after the barium salt had been precipitated a great number of times from dilute hydrochloric acid by barium hydroxide and alcohol alternately that the composition did change slightly. The same result was also observed on digesting the barium salt in dilute acetic acid. After treating in the above manner, barium salts of the following composition were obtained:

- I. C = 7.49; H = 1.63; P = 16.77; Ba = 37.89 per cent.
- II. C = 7.69; H = 1.47; P = 16.75; Ba = 37.72 per cent.
- III. C = 7.26; H = 1.75; P = 16.45; Ba = 36.40 per cent.

These salts were united and dissolved in the least possible amount of 0.5 per cent hydrochloric acid and alcohol added to the solution until a faint permanent turbidity remained, which was just cleared up by the addition of a few drops of dilute hydrochloric acid. The solution was then allowed to stand at room temperature for about two days. There separated slowly a heavy white crust on the bottom of the flask. Under the microscope this showed no definite crystalline structure. The substance was filtered off, washed thoroughly in water, alcohol and ether and dried in the air. It was free from chlorides and gave no reaction with ammonium molybdate. For analysis it was dried at 105° in vacuum over phosphorus pentoxide.

Found: C = 6.07; H = 1.35; P = 16.77; Ba = 40.00; H<sub>2</sub>O = 10.08 per cent.

The carbon found is undoubtedly somewhat too low as this heavy, compact substance burned with extreme difficulty.

The filtrate from the above was precipitated with alcohol, the precipitate filtered, washed and dried in vacuum over sulphuric acid. The following result was obtained on analysis.

Found: C = 7.63; H = 1.57; P = 16.53; Ba = 36.92 per cent.

The heavy, crust-like substance was recrystallized as follows: It was dissolved in dilute hydrochloric acid, filtered and about an equal volume of alcohol added and the mixture allowed to stand over night. The precipitate which was amorphous at first had

then changed into a crystalline form. Under the microscope it appeared as very small globules consisting of microscopic needles. It was filtered, washed free of chlorides in dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid. It was a light, voluminous, snow-white crystalline powder. It was analyzed after drying at  $105^{\circ}$  in vacuum over phosphorus pentoxide.

Found: C = 6.60; H = 1.50; P = 17.33; Ba = 37.45;  $H_2O \approx 8.72$  per cent.

For tri-barium inosite hexaphosphate,  $C_6H_{12}O_{24}P_6Ba_3 = 1066$ :

Calculated, C = 6.75; H = 1.12; P = 17.44; Ba = 33.65 per cent.

The amorphous preparations which remained were united (total weight, 52.5 grams). The substance was rubbed up in a mortar with a small quantity of cold water. A considerable portion of the substance dissolved. The insoluble portion was perfectly white and opaque, but it soon changed into a semi-crystalline form and appeared translucent. After standing for some time it was filtered and washed in water, and finally in alcohol and ether and dried in vacuum over sulphuric acid. When dry it was again treated with water in the same way. These operations were repeated three times.

The filtrates and washings from the above were precipitated by the addition of alcohol and these precipitates reserved for examination, as will be described later.

The water-insoluble substance was dissolved in the least possible quantity of dilute hydrochloric acid (about 5 per cent strength), the free acid was then nearly neutralized with barium hydroxide, the solution filtered and alcohol added until a faint permanent turbidity remained. A concentrated solution of 20 grams of barium chloride was then added and the whole allowed to stand. The substance soon began to crystallize in the same crystal-form as the barium salt from cottonseed meal, viz., in globular masses or bundles of fine microscopic needles. After standing over night the crystals were filtered, washed free of chlorides with water and then in alcohol and ether and allowed to dry in the air. A further crop of the same shaped crystals was obtained from the mother-liquor by carefully adding alcohol and allowing to stand. After filtering, washing and drying these were added to the first crop.

The substance was recrystallized three times in the same way. It was finally obtained as a light snow-white crystalline powder. It weighed about 27 grams.

This was analyzed after drying at  $105^{\circ}$  in vacuum over phosphorus pentoxide.

0.2483 gram substance lost 0.0308 gram  $H_2O$ .

0.2175 gram substance gave 0.0295 gram  $H_2O$  and 0.0507 gram  $CO_2$ .

0.1456 gram substance gave 0.0987 gram  $BaSO_4$  and 0.0864 gram  $Mg_2P_2O_7$ .

Found: C = 6.35; H = 1.51; P = 16.54; Ba = 39.89;  $H_2O$  = 12.40 per cent.

It was again recrystallized in the same manner and the following result obtained on analysis after drying as before:

Found: C = 6.23; H = 1.27; P = 16.17; Ba = 41.48;  $H_2O$  = 12.99 per cent.

For hepta-barium inosite hexaphosphate,  $(C_6H_{11}O_2P_6)_2Ba_7$  = 2267:

Calculated, C = 6.35; H = 0.97; P = 16.40; Ba = 42.39 per cent.

The substance was again dissolved in dilute hydrochloric acid, the solution was filtered and then precipitated by the addition of alcohol. The precipitate was amorphous at first, but on standing in the mother-liquor over night it had changed into the usual crystal-form, but the globules and crystals were much smaller. After filtering and washing free of chlorides in dilute alcohol, alcohol and ether the substance was dried in vacuum over sulphuric acid. It was analyzed after drying at  $105^{\circ}$  in vacuum over phosphorus pentoxide.

0.2320 gram substance gave 0.0325 gram  $H_2O$  and 0.0553 gram  $CO_2$ .

0.1466 gram substance gave 0.0947 gram  $BaSO_4$  and 0.0894 gram  $Mg_2P_2O_7$ .

Found: C = 6.50; H = 1.56; P = 17.00; Ba = 38.01 per cent.

For tri-barium inosite hexaphosphate,  $C_6H_{12}O_2P_6Ba_3$  = 1066:

Calculated, C = 6.75; H = 1.12; P = 17.44; Ba = 38.65 per cent.

As will be noticed from the above analytical results the composition does not change on repeated recrystallizations. We believe therefore that the substance is homogeneous. The variation in the percentage of barium evidently depends upon the formation of more or less acid salts. All the preparations described showed a strong acid reaction on moist litmus paper which indicates free hydrogen ions.

In the estimation of carbon and hydrogen it was found impossible to obtain a white ash by direct combustion even when heated for a long time—the residue in the boat was invariably dark colored, varying from light gray to quite dark. Plimmer and Page<sup>6</sup> also mention the difficulty of completely burning carbon in the presence of phosphoric acid. The crystalline barium salts described in this paper, as well as those from cottonseed meal, are particularly hard to burn; on the other hand the amorphous salts burn more easily. In the combustions of these salts we have always burned the substance twice: first in the regular manner; the dark-colored ash has then been powdered in an agate mortar and mixed in the boat with chromic acid and burned a second time. In this second combustion there has been observed an increase in weight in the carbon dioxide varying from about 1 to 12 mgm. Under these conditions it is impossible to say whether a complete combustion has been effected, and it is not improbable that a small quantity of carbon has escaped oxidation. We are inclined to believe that the percentage of carbon as found is slightly low. As a check upon the carbon content, found in the barium salts we have always prepared and analyzed the free acid, in the combustion of which we have never experienced any serious difficulty—the residue in the boat showing no trace of carbon.

*Preparation of the free acid from the purified crystalline barium salt.*

The acid was prepared from 4 grams of the barium salt in the usual way. After drying in vacuum over sulphuric acid at room temperature it formed a practically colorless thick syrup. Its reactions were identical with those which we reported for the acid from cottonseed meal.<sup>7</sup> For analysis it was dried in vacuum over phosphorus pentoxide at 78°. The preparation darkened perceptibly in color but did not turn black.

0.4936 gram substance gave 0.1366 gram  $H_2O$  and 0.1960 gram  $CO_2$ .

0.1645 gram substance gave 0.1601 gram  $Mg_2P_2O_7$ .

Found: C = 10.82; H = 3.09; P = 27.12 per cent.

For inosite hexaphosphate,  $C_6H_{18}O_{24}P_6$  = 660:

Calculated, C = 10.90; H = 2.72; P = 28.18 per cent.

<sup>6</sup> *Biochem. Journ.*, vii, p. 167, 1913.

<sup>7</sup> *Loc. cit.*

*Preparation of inosite from the barium salt.*

The amorphous barium salt was used. Of the dry salt, 9.3 grams were heated in a sealed tube with 25 cc. of 5 N sulphuric acid to 150°–160° for three hours. After cooling the contents of the tube were very dark in color and some carbonaceous substance had separated, showing that considerable decomposition had taken place. The sulphuric and phosphoric acids were precipitated with excess of barium hydroxide, filtered and washed and the filtrate freed from excess of barium with carbon dioxide. The filtrate was evaporated to small bulk and decolorized with animal charcoal and then evaporated to dryness on the water bath. The residue was taken up in a little hot water, filtered from traces of barium carbonate and the inosite brought to crystallize by the addition of alcohol and ether. It separated in needles free from water of crystallization. After filtering, washing in alcohol and ether and drying in the air it weighed 1.6 grams, which represents a yield of about 75 per cent of the total carbon. For analysis it was recrystallized seven times in the same manner as above and was finally obtained in beautiful colorless needles free from water of crystallization. It melted at 224° uncorrected and gave the reaction of Scherer. It did not lose in weight on drying at 105° in vacuum over phosphorus pentoxide.

0.1442 gram substance gave 0.0878 gram  $H_2O$  and 0.2113 gram  $CO_2$ .

Found: C = 39.96; H = 6.81 per cent.

For  $C_6H_{12}O_6$ , calculated: C = 40.00; H = 6.66 per cent.

*Hydrolysis of the acid with water alone.*

The acid which was used had been prepared from the amorphous barium salt. Two grams of the dry preparation were heated with 25 cc. of water in a sealed tube to 190° for three and one-half hours. After cooling there was no pressure on opening the tube. The content was of dark brown color and a considerable quantity of a black, carbonized substance had separated. The whole was diluted with water and filtered and the phosphoric acid was precipitated with barium hydroxide in excess. The precipitate was filtered off and examined to see if any unchanged barium phytate could be isolated from it. Apparently the acid

had been completely decomposed during the heating as no trace of barium phytate could be found.

The filtrate from the barium phosphate was freed from excess of barium by carbon dioxide and the filtrate evaporated to dryness on the water bath. The residue was a sticky, amber colored syrup. It was taken up in a small amount of water and washed into an Erlenmeyer flask. On the addition of alcohol the solution turned cloudy but it could not be brought to crystallize by repeated scratching with a glass rod. It was allowed to stand over night when an amber colored, syrupy layer had separated on the bottom. The upper portion of the liquid was poured off and mixed with ether. On standing a further quantity of amber colored syrup had separated. The liquid was decanted and evaporated on the water bath until a small syrupy residue remained. On scratching with a glass rod a substance began to crystallize in small prisms. The other syrups were made to crystallize in the same manner. They were then extracted several times with small quantities of alcohol. The residues were then dissolved in hot water and crystallized by the addition of alcohol. After recrystallizing three times it was obtained in small colorless needles. It weighed 0.25 gram. It melted at  $222^{\circ}$  uncorrected and gave the Scherer reaction and was therefore undoubtedly inosite. After recrystallizing it again melted at  $222^{\circ}$  uncorrected. The crystals did not contain water of crystallization as there was no loss in weight on drying at  $105^{\circ}$  in vacuum for one hour. The substance was further identified as inosite by the analysis.

0.1019 gram substance gave 0.0629 gram  $H_2O$  and 0.1492 gram  $CO_2$ .

Found: C = 39.93; H = 6.90 per cent.

For  $C_6H_{12}O_6$ , calculated: C = 40.00; H = 6.66 per cent.

The alcoholic washings from above and the mother liquor on evaporation left a dark-colored, non-crystallizable syrup. This syrup strongly reduced Fehling's solution on boiling.

It is noteworthy that the amount of inosite obtained by cleavage with water is much less than when dilute sulphuric acid is used. The amount of inosite isolated above represents only about one-half the quantity obtained when the hydrolysis is effected in the presence of acid. It is possible, however, that in this case the inosite had been less completely isolated since the adhering syrupy substance rendered crystallization more difficult.

*Examination of the water-soluble portion of the amorphous barium salt.*

As has been already mentioned on page 158 the filtrates containing the water-soluble portion of the amorphous barium salt were precipitated with alcohol. After filtering and washing the precipitate was dried in vacuum over sulphuric acid. It was again digested in water three times and filtered from small insoluble matter and the filtrates precipitated with alcohol and dried. It was finally obtained as a snow-white amorphous powder. As it was impossible to obtain any crystalline substance from it, it was analyzed after drying at  $105^{\circ}$  in vacuum over phosphorus pentoxide.

0.3504 gram substance lost 0.0294 gram  $H_2O$  on drying.

0.3210 gram substance gave 0.0616 gram  $H_2O$  and 0.1342 gram  $CO_2$ .

0.2415 gram substance gave 0.1465 gram  $BaSO_4$  and 0.1236 gram  $Mg_2P_2O_7$ .

Found: C = 11.40; H = 2.14; P = 14.26; Ba = 35.69;  $H_2O$  = 8.39 per cent.

The substance was free from inorganic phosphate and chlorides, and bases other than barium could not be detected. We hope to investigate this substance further.



## CONCERNING PHYTIN IN CORN.

SEVENTH PAPER ON PHYTIN.

By R. J. ANDERSON.

(From the Chemical Laboratory of the New York Agricultural Experiment Station, Geneva, N. Y.)

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The organic phosphoric acid compound occurring in corn has been particularly studied by Vorbrodt.<sup>1</sup> In an exhaustive treatise on the subject he reports analytical results obtained from crystalline barium salts which led him to believe that the substance was different from phytin. The barium salt corresponded to the formula  $C_{12}H_{26}O_{46}P_{11}Ba_7$  according to which the formula of the acid would be  $C_{12}H_{40}O_{46}P_{11}$ . Vorbrodt also showed that the substance gave inosite and phosphoric acid on cleavage either with dilute sulphuric acid or water alone in neutral solution. The same subject has also been investigated by Hart and Totingham.<sup>2</sup> They report the preparation and analysis of the free acid. The analytical data agree very closely with those required for phytic acid and they concluded that corn contains phytin. They also showed that the acid yields inosite on hydrolysis in a sealed tube in the presence of dilute sulphuric acid.

We have undertaken to reexamine this substance in the hope of identifying it either with phytic acid or the compounds which we have shown to exist in cottonseed meal<sup>3</sup> and oats.<sup>4</sup> At first we were unable to obtain the barium salt in crystalline form but the amorphous salt gave results on analysis which approximately agreed with the corresponding barium phytate. The free acid, prepared from this amorphous compound, gave about 1 per cent

<sup>1</sup> *Anzeiger Akad. Wiss. Krakau*, 1910, Series A, p. 484.

<sup>2</sup> *Research Bulletin 9*, Univ. of Wisconsin Agric. Exp. Station, 1910.

<sup>3</sup> *This Journal*, xiii, p. 311, 1912; *Tech. Bulletin 25*, N. Y. Agric. Exp. Station, 1912, and also a preceding article.

<sup>4</sup> See preceding article.

too high carbon and about 0.8 per cent too high phosphorus. We finally succeeded, however, in preparing a crystalline barium salt. It was purified by repeated recrystallizations until the composition remained constant. The product was free from inorganic phosphate and it did not contain a determinable quantity of bases other than barium. Judging by crystal-form, composition and properties the substance is identical with those previously isolated from cottonseed meal and oats.

The analytical results obtained from these purified crystalline barium salts do not agree with the formula proposed by Vorbrodt. We find the phosphorus over 1 per cent higher and the relation between carbon and phosphorus is as 1 : 1. The phosphorus content is also considerably higher than that required for a corresponding salt calculated on the usual phytic acid formula.

The barium salt analyzed by Vorbrodt had been prepared from the previously isolated acid by partially neutralizing with barium hydroxide and concentrating in vacuum. The crystalline salt which then separated was washed, dried and analyzed. Apparently no attempt had been made to recrystallize it and it is probable that the substance had contained small quantities of impurities which might be sufficient to account for the difference in analytical results between this product and the repeatedly recrystallized salts which we have analyzed.

The composition of the purified salts described in this paper agrees more closely with salts of inosite hexaphosphate than with salts calculated on the basis of the usual phytic acid formula.

#### EXPERIMENTAL PART.

##### *Isolation of the substance from corn.*

The corn used in these experiments was the ordinary cornmeal used as cattle feed at this station. Ground corn-meal, 3500 grams, was digested in 7 liters of 0.2 per cent hydrochloric acid over night. It was then strained and filtered and the clear amber-colored filtrate precipitated by adding about one and one-half volumes of alcohol. After settling, the precipitate was filtered and washed in dilute alcohol. The precipitate was then dissolved in a small amount of 0.5 per cent hydrochloric acid and filtered from insoluble matter. This acid solution gave only a

very slight precipitate on the addition of alcohol. The substance was, therefore, transformed into a barium salt by precipitating with barium hydroxide to slight alkaline reaction. After heating on the water bath for some time the precipitate was filtered and washed in water. It was again dissolved in 0.5 per cent hydrochloric acid, filtered and reprecipitated with barium hydroxide. After standing over night the precipitate was filtered and washed thoroughly in water. The substance was again dissolved in 0.5 per cent hydrochloric acid, filtered and then precipitated by the addition of an equal volume of alcohol. The precipitate after settling was filtered and washed in dilute alcohol. The substance was then precipitated three times more in the same manner and after finally filtering, washing in dilute alcohol, alcohol and ether, it was dried in vacuum over sulphuric acid. A white amorphous powder was obtained which weighed 11.8 grams. The substance gave no reaction for chlorides. The dilute nitric acid solution gave no reaction with ammonium molybdate after warming for some time.

The following results were obtained on analysis after drying at  $105^{\circ}$  in vacuum over phosphorus pentoxide to constant weight.

Found: C = 7.25; H = 1.51; P = 16.65; Ba = 37.11 per cent.

The carbon is somewhat high, otherwise the result agrees with the calculated percentages for tri-barium phytate,  $C_6H_{18}O_{27}P_6Ba_3$ :

Calculated, C = 6.42; H = 1.60; P = 16.60; Ba = 36.78 per cent.

*Preparation of the free acid from the above amorphous barium salt.*

The acid was prepared from 3 grams of the barium salt in the usual way, *i.e.*, the barium was precipitated with slight excess of sulphuric acid, filtered and the filtrate precipitated with copper acetate. The copper precipitate was filtered and washed thoroughly in water, suspended in water and decomposed with hydrogen sulphide, filtered and the filtrate evaporated in vacuum at a temperature of  $40^{\circ}$ – $45^{\circ}$  to a syrupy consistency and finally dried in vacuum over sulphuric acid. The product was a thick, faintly amber colored syrup. For analysis it was dried at  $105^{\circ}$  in vacuum over phosphorus pentoxide. It turned very dark in color.

Found: C = 11.09; H = 3.04; P = 26.85 per cent.

Both carbon and phosphorus are higher than required for phytic acid,  $C_6H_{24}O_{27}P_6$ :

Calculated, C = 10.08; H = 3.36; P = 26.05 per cent.

*Preparation of the substance from corn as a crystalline barium salt.*

A larger quantity of corn-meal was extracted with 0.2 per cent hydrochloric acid, the extract filtered and precipitated by adding a concentrated solution of barium chloride. The precipitate was then purified and crystallized in the manner described for cottonseed meal in the preceding article. After the substance had been separated from dilute hydrochloric acid solutions twelve times (eleven times in crystalline form), it was obtained as a beautiful snow-white, bulky crystalline powder which weighed 49 grams. The crystalline form was identical with that of the barium salts from cottonseed meal and oats, *i.e.*, globular masses of microscopic needles. The substance was free from chlorides and inorganic phosphate and we were unable to detect any metals other than barium.

For analysis it was dried at  $105^\circ$  in vacuum over phosphorus pentoxide.

0.4101 grams substance gave 0.0607 gram  $H_2O$  and 0.0985 gram  $CO_2$ .

0.1477 gram substance gave 0.1035 gram  $BaSO_4$  and 0.0860 gram  $Mg_2P_2O_7$ .

Found: C = 6.55; H = 1.65; P = 16.23; Ba = 41.23 per cent.

A portion of this salt was recrystallized as follows: 5 grams were dissolved in a small quantity of 3 per cent hydrochloric acid; barium hydroxide was carefully added until a slight permanent precipitate remained; the solution was filtered and a concentrated solution of 2 grams of barium chloride added. The perfectly clear solution was allowed to stand at room temperature for about two days when the substance separated slowly in the usual form. It was filtered, washed free of chlorides with water and then in alcohol and ether and dried in the air. Yield, 4.5 grams. The substance gave no reaction with ammonium molybdate. It was analyzed after drying at  $105^\circ$  in vacuum over phosphorus pentoxide.

0.4770 gram substance lost 0.0561 gram  $H_2O$ .

0.1767 gram substance lost 0.0209 gram  $H_2O$ .

0.4209 gram substance gave 0.0491 gram  $\text{H}_2\text{O}$  and 0.0931 gram  $\text{CO}_2$ .  
 0.3616 gram substance gave 0.0427 gram  $\text{H}_2\text{O}$  and 0.0832 gram  $\text{CO}_2$ .  
 0.1553 gram substance gave 0.1110 gram  $\text{BaSO}_4$  and 0.0907 gram  $\text{Mg}_2\text{P}_2\text{O}_7$ .  
 Found: I. C = 6.03; H = 1.30; P = 16.28; Ba = 42.06 per cent.  
 II. C = 6.27; H = 1.32;  $\text{H}_2\text{O}$  = 11.76 and 11.82 per cent.  
 For hepta-barium inosite hexaphosphate  $(\text{C}_6\text{H}_{11}\text{O}_{24}\text{P}_6)_2\text{Ba}_7 = 2267$ :  
 Calculated, C = 6.35; H = 0.97; P = 16.40; Ba = 42.39 per cent.  
 For  $16\text{H}_2\text{O}$  calculated: 11.27 per cent.

This recrystallized salt was again recrystallized as follows: It was dissolved in a small quantity of 3 per cent hydrochloric acid, filtered and diluted with a small quantity of water. Alcohol was then added until a faint permanent cloudiness remained. On standing at room temperature the substance soon began to crystallize in the usual form except that the crystals were much smaller. After standing over night it was filtered, washed in dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid. The product was a snow-white fine crystalline powder. It gave no reaction for chlorides and none for inorganic phosphate. It was analyzed after drying at  $105^\circ$  in vacuum over phosphorus pentoxide.

0.3719 gram substance gave 0.0465 gram  $\text{H}_2\text{O}$  and 0.0887 gram  $\text{CO}_2$ .  
 0.1780 gram substance gave 0.1187 gram  $\text{BaSO}_4$  and 0.1091 gram  $\text{Mg}_2\text{P}_2\text{O}_7$ .  
 Found: C = 6.50; H = 1.40; P = 17.08; Ba = 39.14 per cent.  
 For tri-barium inosite hexaphosphate,  $\text{C}_6\text{H}_{12}\text{O}_{24}\text{P}_6\text{Ba}_3 = 1066$ :  
 Calculated, C = 6.75; H = 1.12; P = 17.44; Ba = 38.65 per cent.

#### *Preparation of the free acid.*

The acid was prepared in the usual way from about 4 grams of the crystalline barium salt. After drying in vacuum over sulphuric acid it was obtained as a thick, very faintly amber colored syrup. In appearance and reactions it corresponded exactly with the acids prepared from the crystalline barium salts which have been described previously.

A portion of the above dried acid dissolved in water and acidified with nitric acid gave no precipitate after warming for some time with ammonium molybdate solution.

For analysis the preparation was dried first for ten days in vacuum over sulphuric acid at room temperature and then at  $78^\circ$  in vacuum over phosphorus pentoxide to constant weight.

The color did not change by drying in vacuum at room temperature but at 78° the color darkened somewhat.

0.3405 gram substance gave 0.0919 gram  $H_2O$  and 0.1356 gram  $CO_2$ .

0.1796 gram substance gave 0.1754 gram  $Mg_2P_2O_7$ .

Found: C = 10.86; H = 3.02; P = 27.22 per cent.

For inosite hexaphosphate,  $C_6H_{13}O_{21}P_6 = 660$ :

Calculated, C = 10.90; H = 2.72; P = 28.18 per cent.

For phytic acid,  $C_6H_{24}O_{27}P_6 = 714$ :

Calculated, C = 10.08; H = 3.36; P = 26.05 per cent.

# A CONTRIBUTION TO THE CHEMISTRY OF PHYTIN.

## I. COMPOSITION OF BARIUM PHYTATE AND PHYTIC ACID.

## II. A STUDY OF THE PROPERTIES OF PHYTIC ACID AND ITS DECOMPOSITION PRODUCTS.

EIGHTH PAPER ON PHYTIN.

By R. J. ANDERSON.

(From the Chemical Laboratory of the New York Agricultural Experiment Station, Geneva, N. Y.)

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In previous reports from this laboratory it has been shown that the organic phosphoric acids existing in cottonseed meal,<sup>1</sup> oats<sup>2</sup> and corn<sup>3</sup> yield identical crystalline barium salts which differ in composition from the corresponding, so called, barium phytates. The free organic phosphoric acids, isolated from these crystalline barium salts, although identical so far as analysis is concerned, differ in composition from phytic acid. These crystalline salts had all been carefully purified by repeated recrystallizations and it appears, therefore, reasonable to believe that they were purer than any previously described barium phytate. In so far as we may judge by crystal-form, composition and reactions the above compounds are identical and are salts of an acid of the formula,  $C_2H_6O_8P_2$  or  $C_6H_{18}O_{24}P_6$ . If the latter be the correct formula, which appears probable, then it differs from the phytic acid formula of Neuberg,<sup>4</sup>  $C_6H_{24}O_{27}P_6$ , by three molecules of water which is also the difference between phytic acid and inosite hexaphosphate.

Previously we have reported<sup>5</sup> numerous salts of phytic acid prepared from commercial phytin. These salts, however, were

<sup>1</sup> This *Journal*, xiii, p. 311, 1912; N. Y. Agric. Exp. Station, Tech. Bulletin 25, 1912; also a preceding article.

<sup>2</sup> See preceding article.

<sup>3</sup> See preceding article.

<sup>4</sup> *Biochem. Zeitschr.*, ix, pp. 551 and 557, 1908.

<sup>5</sup> This *Journal*, xi, p. 471, 1912; xii, p. 97, 1912; N. Y. Agric. Exp. Station, Tech. Bulletin 19 and 21, 1912.

mostly amorphous and particularly the barium salts, with one exception, were not obtained in crystalline form. These amorphous compounds gave results on analysis which corresponded closely with percentages calculated on the basis of the usual formula for phytic acid, viz.,  $C_6H_{24}O_{27}P_6$ . In dealing with amorphous substances, however, some doubt may be felt as to their being homogeneous products.

Since the crystalline salts, mentioned above, differ in composition from compounds calculated on the usual formula for phytic acid, we are forced to the conclusion either that the organic phosphoric acid existing in cottonseed meal, oats and corn is different and distinct from phytic acid or that the formula of phytic acid itself is wrong, having possibly been based upon analytical data of somewhat impure preparations.

It seemed of importance to determine whether any real difference exists between the barium salts of phytic acid prepared from commercial phytin and the crystalline salts obtained from cottonseed meal, oats and corn. We have, therefore, reexamined the commercial phytin using some of the same preparation as before. After carefully purifying the barium salt of the substance we found that it crystallized very readily and no difference can be observed either in crystal-form, composition or reactions, of the salts prepared in this way, from the crystalline salts previously referred to. All of these compounds are therefore identical and the analytical data indicate that they are salts of the acid  $C_2H_6O_8P_2$  or  $C_6H_{18}O_{24}P_6$ .

The composition, as determined by analysis, of the free acid prepared from the crystalline barium phytate also agrees more closely with the above formulas than with the usual formula of phytic acid  $C_6H_{24}O_{27}P_6$ . The phosphorus was found too low in this case as well as in the acids previously described. This, however, is undoubtedly due to the fact that the acid is largely hydrolyzed on drying.

It appears very probable then that the organic phosphoric acid described above and known as phytic acid is either inositol hexaphosphate,  $C_6H_{18}O_{24}P_6$ , or else an isomer of the same. We have, however, no direct information concerning the molecular magnitude of the acid.

We have endeavored to prepare a neutral ester of the acid with

which molecular weight determinations might be made but so far these attempts have failed. By acting on the silver salt of the acid, suspended in absolute methyl alcohol, with methyl iodide an ester is formed but it apparently suffers partial decomposition in drying. Moreover, we have been unable to prepare a neutral silver salt. Only acid silver salts have been obtained even from solutions of phytic acid neutralized with ammonia. From such salts, naturally, only acid esters could be obtained.

As has been pointed out by Starkenstein,<sup>6</sup> which observation we have confirmed,<sup>7</sup> apparently only one-half of the acid hydroxyls of phytic acid are particularly reactive. Some of the acid hydroxyls appear to be very weak. It is no doubt due to this fact that from a neutral solution of ammonium phytate only acid silver salts are precipitated with silver nitrate.

Starkenstein<sup>8</sup> reported that the commercial phytin which he had examined contained relatively large quantities of inorganic phosphate and that it also contained free inosite and he concluded that the substance undergoes spontaneous decomposition. He also found that after drying the preparation at 100° the greater portion of the phosphorus was present as inorganic phosphate.

These results, so far as the formation of inosite on mere drying is concerned, we could not confirm<sup>9</sup> in the phytin preparations which we had on hand. From a sample of commercial phytin which had been in our laboratory for several years we could not isolate a trace of inosite either before or after drying at 115°. At that time we made no effort to determine the increase in inorganic phosphate on drying at 100° or higher. Observations made since then, however, have shown without any doubt that phytin undergoes spontaneous decomposition when kept under ordinary conditions at room temperature. Both the salts and the free acid decompose slowly, with liberation of inorganic phosphate. The free acid decomposes much faster than the salts. We have also found that a very perceptible increase in inorganic phosphate

<sup>6</sup> *Biochem. Zeitschr.*, xxx, p. 65, 1910.

<sup>7</sup> *This Journal*, xi, p. 475, 1912; *Tech. Bulletin* 19, N. Y. Agric. Exp. Station, 1912.

<sup>8</sup> *Loc. cit.*, pp. 59 and 60.

<sup>9</sup> *This Journal*, xi, p. 473, 1912; *Tech. Bulletin* 19, N. Y. Agric. Exp. Station, 1912.

occurs on drying at  $105^{\circ}$  in vacuum. In this case also the free acid decomposes to a greater extent than the salts.

Although notable quantities of inorganic phosphoric acid are liberated from phytic acid and its salts under the above conditions, we have again been unable to demonstrate the presence of inosite as one of the spontaneous decomposition products. In this connection we especially examined a specimen of phytic acid which had been kept in the laboratory for about eighteen months. The preparation had been kept in a glass-stoppered bottle at ordinary temperature but at no time had it been exposed to direct sunlight. When first prepared the acid was a practically colorless, thick syrup containing about 20 per cent of water and it gave no reaction with ammonium molybdate. It darkened gradually in color and when examined the color was quite black. Analysis showed that about one-eighth of the total phosphorus was present in the form of inorganic phosphoric acid. A quantity of this preparation corresponding to 10 grams of the dry acid was examined for inosite but no trace of this substance could be found although the preparation should have contained about 0.3 gram of inosite had the organic radical corresponding to the free inorganic phosphoric acid present separated in the form of inosite.

Since the organic part of the phytic acid radical had not separated as inosite under the above conditions of spontaneous decomposition it appeared of interest to determine, if possible, what product or products had been formed and in what manner the decomposition had occurred. While we are unable to answer these questions fully at this time, the results would indicate that, under the above conditions, the phytic acid undergoes only partial decomposition with formation of penta- or tetra-phosphoric acid esters of inosite and free phosphoric acid.

The aqueous solution of the above partially decomposed phytic acid was precipitated with barium hydroxide. The barium precipitate was freed from inorganic phosphate in our usual way, *i.e.*, by precipitating its dilute hydrochloric acid solution with alcohol until the product gave no reaction with ammonium molybdate reagent. The final product was a white, amorphous powder. We succeeded in separating this substance into two portions, one a crystalline salt showing all the characteristics and composition of unchanged barium phytate and a second amorphous portion

which, judging by analysis, was probably a mixture of the barium salts of inosite penta- and tetra-phosphate.

Since no free inosite could be isolated from this preparation although it had been standing for eighteen months and had undergone considerable decomposition it would seem that a very long time would be required for complete decomposition, *i.e.*, until free inosite were present. On drying a sample of the same acid at  $105^{\circ}$  for forty-eight hours under diminished pressure, however, the decomposition products isolated were found to be quite different. In this case we found that about 75 per cent of the phosphorus was present as inorganic phosphoric acid and we were unable to isolate any unchanged barium phytate. Apparently all of the phytic acid had been partially decomposed and some of it completely, for we obtained 0.25 gram of inosite from 10 grams of the acid after drying as above. The organic phosphoric acid or acids remaining undecomposed were isolated as barium salts. None of these, however, could be obtained in pure form. But by taking advantage of their varying solubilities in water and mixtures of acidulated water and alcohol we were able to separate it into four fractions. All of these fractions had different composition and it would appear probable that they represent more or less impure mixtures of the barium salts of tetra- tri- di- and mono-phosphoric acid esters of inosite.

When phytic acid has been completely dried at temperatures ranging from  $60^{\circ}$  to  $105^{\circ}$  we have noticed that it is not completely soluble in water. Some insoluble substance separates in thin gelatinous plates. We have not been able to obtain a sufficient quantity of this substance for a complete examination. Judging by the analysis of one small sample it is a complex decomposition product of phytic acid and possibly a partially dehydrated tri-phosphoric acid ester of inosite.

#### EXPERIMENTAL PART.

##### *Preparation of the crystallized barium phytate.*

A sample of the same commercial phytin as formerly examined was transformed into the barium salt as follows: 50 grams of the substance were suspended in about 1500 cc. of water and dissolved by the careful addition of dilute hydrochloric acid. Barium hydroxide

was then added to slight alkaline reaction and the whole allowed to stand over night. The barium hydroxide used was Kahlbaum's c.p. which had been recrystallized. The precipitate was filtered and washed thoroughly in water. It was dissolved in the minimum quantity of about 3 per cent hydrochloric acid, filtered and again precipitated with barium hydroxide. These operations were repeated four times. The substance was then precipitated from the same strength hydrochloric acid with alcohol. After thoroughly washing the precipitate with dilute alcohol it was again dissolved in 3 per cent hydrochloric acid and precipitated a fifth time with barium hydroxide. The dilute hydrochloric acid solution of the substance was then twice precipitated with alcohol. After finally filtering, the precipitate was washed free of chlorides with dilute alcohol and then washed in alcohol and ether and dried in vacuum over sulphuric acid. The substance was then a snow-white amorphous powder. The dry powder was rubbed up in a mortar with a small quantity of cold water. The insoluble portion changed into a semicrystalline form after a short time. This was filtered and washed thoroughly in water. It was dissolved in the minimum quantity of 3 per cent hydrochloric acid. A dilute solution of barium hydroxide was then added until a slight permanent precipitate remained which was nearly cleared up by the careful addition of dilute hydrochloric acid. The solution was filtered and allowed to stand over night. The substance soon began to separate in crystalline form. Under the microscope it appeared perfectly homogeneous and the crystal-form was identical with that observed with the barium salts from cottonseed meal, oats and corn, *i.e.*, the substance crystallized in globular masses of microscopic needles. The substance was filtered off and washed free of chlorides with water and then in alcohol and ether and dried in vacuum over sulphuric acid.

To the mother-liquor a concentrated solution of 15 grams of barium chloride was added and allowed to stand for another twenty-four hours. A further quantity of the same shaped crystals had then separated which were filtered, washed and dried as above.

The two crystalline portions were united and recrystallized in the same manner and again dried in vacuum over sulphuric acid. It was then dissolved in the same strength hydrochloric acid and

precipitated by adding an equal volume of alcohol. The precipitate was amorphous at first but after standing a few hours it had changed into the crystalline form—identical with the above but the crystals were much smaller. After standing over night it was filtered, washed free of chlorides with dilute alcohol and then in alcohol and ether and dried in vacuum over sulphuric acid. The product was a snow-white, light, bulky crystalline powder. It weighed 24 grams. It was free from chlorides and inorganic phosphate. In 0.5 gram of the substance no bases other than barium could be detected.

For analysis it was dried at  $105^{\circ}$  in vacuum over phosphorus pentoxide.

0.2931 gram substance gave 0.0365 gram  $H_2O$  and 0.0714 gram  $CO_2$ .

0.1743 gram substance gave 0.1144 gram  $BaSO_4$  and 0.1073 gram  $Mg_2P_2O_7$ .

Found: C = 6.64; H = 1.39; P = 17.15; Ba = 38.62 per cent.

For tri-barium inosite hexaphosphate,  $C_6H_{12}O_{24}P_6 Ba_3 = 1066$ :

Calculated, C = 6.75; H = 1.12; P = 17.44; Ba = 38.65 per cent.

The above salt was recrystallized as follows: 5 grams were dissolved in the least possible quantity of about 3 per cent hydrochloric acid and the free acid nearly neutralized by the careful addition of barium hydroxide until a faint permanent precipitate remained. The solution was then allowed to stand over night. The substance had then separated in the same crystalline form as before. It was filtered, washed free of chlorides with water and then in alcohol and ether and allowed to dry in the air.

The product was a heavy, crystalline, snow-white powder. Its dilute nitric acid solution gave no reaction with ammonium molybdate.

For analysis it was dried in vacuum over phosphorus pentoxide at  $105^{\circ}$ .

0.5772 gram substance gave 0.0642 gram  $H_2O$ .

0.2124 gram substance gave 0.0234 gram  $H_2O$ .

0.5130 gram substance gave 0.0547 gram  $H_2O$  and 0.1180 gram  $CO_2$ .

0.1887 gram substance gave 0.1348 gram  $BaSO_4$  and 0.1098 gram  $Mg_2P_2O_7$ .

Found: C = 6.27; H = 1.19; P = 16.22; Ba = 42.03;  $H_2O$  = 11.12 and 11.01 per cent.

For hepta-barium inosite hexaphosphate,  $(C_6H_{11}O_{24}P_6)_2Ba_7$  or  $C_{12}H_{22}O_{48}P_{12}Ba_7 = 2267$ :

Calculated, C = 6.35; H = 0.97; P = 16.40; Ba = 42.39 per cent.

For  $16H_2O$ , calculated = 11.27 per cent.

*Preparation of the free acid.*

The acid was prepared from 5 grams of the first crystalline barium salt in the usual way, *i.e.*, the substance was suspended in water and the barium removed by a slight excess of dilute sulphuric acid, filtered and the filtrate precipitated with excess of copper acetate. The copper precipitate was filtered, washed thoroughly in water, suspended in water and the copper removed with hydrogen sulphide. The filtrate was then evaporated to small bulk in vacuum at a temperature of 40°–45° and finally dried in vacuum over sulphuric acid. There remained a practically colorless, thick syrup. The dilute aqueous solution of the acid gave no reaction for inorganic phosphoric acid with ammonium molybdate; the concentrated aqueous solution gave a pure white, crystalline precipitate with ammonium molybdate which, standing at room temperature, remained unchanged for many months but which quickly turned yellowish in color on heating. The reactions of the acid with bases were identical with those previously reported.

For analysis it was dried in vacuum over phosphorus pentoxide to constant weight at the temperature of boiling alcohol. In drying at this temperature the color darkened somewhat.

0.3830 gram substance gave 0.1106 gram  $H_2O$  and 0.1517 gram  $CO_2$ .

0.1839 gram substance gave 0.1802 gram  $Mg_2P_2O_7$ .

Found: C = 10.80; H = 3.23; P = 27.31 per cent.

For inosite hexaphosphate,  $C_6H_{18}O_{21}P_6 = 660$ :

Calculated, C = 10.90; H = 2.72; P = 28.18 per cent.

For phytic acid, according to Neuberg,  $C_6H_{24}O_{27}P_6 = 714$ :

Calculated, C = 10.08; H = 3.36; P = 26.05 per cent.

*Concerning some chemical properties of phytic acid. Spontaneous liberation of inorganic phosphoric acid at ordinary temperature and on drying.*

Freshly prepared phytic acid is a practically colorless syrup—especially when, in the concentration of its aqueous solution, the temperature is not allowed to rise above 50°. When the acid has been prepared from a pure salt, free from inorganic phosphate, the free acid does not give any reaction with ammonium molybdate for inorganic phosphoric acid. Whenever such colorless specimens of phytic acid are preserved for any length of time the color always

darkens. The change in color is more rapid when the concentrated aqueous solution is allowed to stand exposed to the air or preserved in a well stoppered bottle than when the acid is kept in the desiccator but even under the latter condition the color gradually deepens over light yellow, deep yellow, light brown and finally after several months the color is dark brown or black. When the acid is dried for analysis either in vacuum or in an air bath the color darkens very materially in a short time, especially when dried at  $100^{\circ}$  or higher. When dried at a temperature of  $60^{\circ}$  or  $78^{\circ}$  in vacuum the color darkens somewhat but very slightly in comparison with that produced at higher temperatures.

Patten and Hart<sup>10</sup> asserted that the acid turned dark in color on drying at  $110^{\circ}$  without undergoing any decomposition. As mentioned by Vorbrodt<sup>11</sup> the grounds for this statement are not quite clear. A striking change in color such as phytic acid suffers in drying or on mere keeping either in the desiccator or under ordinary conditions would very likely indicate a more or less serious decomposition.

In order to determine to what extent decomposition occurs it was decided to make a series of inorganic phosphoric acid determinations by the usual molybdate method on phytic acid preparations before and after drying. While absolute accuracy could hardly be expected or claimed for this method, at least comparable results would be obtained when the precipitations were done under similar conditions.

One portion of the acid was dried at  $105^{\circ}$  in vacuum over phosphorus pentoxide to constant weight. It was then dissolved in water, neutralized with ammonia, acidified with nitric acid, ammonium nitrate added and heated to  $65^{\circ}$ . Ammonium molybdate was then added and kept at the above temperature for one hour. The precipitate was then determined as magnesium pyrophosphate in the usual way.

Another portion was treated in the same manner without drying. The amount of moisture found on drying as above was deducted from the weight taken.

<sup>10</sup> *Amer. Chem. Journ.*, xxxi, p. 570, 1904.

<sup>11</sup> *Anzeiger Akad. Wiss. Krakau*, 1910, Series A, p. 484.

The acid analyzed as reported on p. 178 was used for the first determinations. The fresh preparation, dried in vacuum over sulphuric acid as described, contained about 15 per cent of water and it gave no reaction with ammonium molybdate. It was allowed to stand in the laboratory at summer temperature (about 80° or 90° F.) in a loosely covered dish for three or four weeks. The color had then changed to light brown. On drying at 105° in vacuum over phosphorus pentoxide for about twenty-four hours to constant weight it lost about 22 per cent of its weight, showing that it had absorbed about 7 per cent of water during this time.

The acid (p. 178) contained 27.31 per cent of phosphorus.

The dried preparation gave the following as inorganic phosphate: 0.2508 gram dry substance gave 0.0696 gram  $\text{Mg}_2\text{P}_2\text{O}_7$ , equivalent to 7.73 per cent phosphorus or 28.30 per cent of the total phosphorus was precipitated as inorganic phosphoric acid.

Before drying: 0.1889 gram (dry substance calculated) gave 0.0039 gram  $\text{Mg}_2\text{P}_2\text{O}_7$ , equivalent to 0.57 per cent of phosphorus or 2.08 per cent of total phosphorus.

As will be noticed from the above figures 26.2 per cent of the total phosphorus had been hydrolyzed by drying at 105° for about twenty-four hours.

An old sample of phytic acid which had been kept in the laboratory for about eighteen months was examined in the same manner. It was practically black in color. It lost about 22 per cent of its weight on drying as above for about twenty hours. After decomposing by the Neumann method it was found to contain 27.68 per cent of phosphorus.

The dried preparation gave the following:

0.2348 gram dry substance gave 0.0733 gram  $\text{Mg}_2\text{P}_2\text{O}_7$ , equivalent to 8.70 per cent of phosphorus or 31.43 per cent of the total phosphorus was present as inorganic phosphoric acid.

Before drying: 0.2651 gram (dry substance calculated) gave 0.0295 gram  $\text{Mg}_2\text{P}_2\text{O}_7$ , equivalent to 3.10 per cent of phosphorus or 11.19 per cent of the total phosphorus had been hydrolyzed in about eighteen months under ordinary room conditions.

In the above case about 20.2 per cent of the total phosphorus had been hydrolyzed on drying at 105° for about twenty hours.

A sample of the pure recrystallized barium phytate was examined for inorganic phosphoric acid in the same way. The fresh preparation gave no reaction with ammonium molybdate. After standing in the laboratory for five or six weeks the following results were obtained:

After drying at 105° in vacuum over phosphorus pentoxide 0.2108 gram substance gave 0.0104 gram  $\text{Mg}_2\text{P}_2\text{O}_7$ , equivalent to 1.37 per cent of phosphorus.

Before drying: 0.2060 gram (dry substance calculated) gave 0.0030 gram  $\text{Mg}_2\text{P}_2\text{O}_7$ , equivalent to 0.40 per cent of phosphorus.

By drying at 105° the inorganic phosphorus increased about two and one-half times.

A portion of the inorganic phosphoric acid found in the above determinations was probably due to cleavage of the phytic acid by the dilute nitric acid. Such cleavage appears to take place slowly and uniformly as shown by the following experiment:

Another portion (0.1876 gram substance) of the same barium phytate without previous drying gave 0.0022 gram  $\text{Mg}_2\text{P}_2\text{O}_7$  after heating one hour with the ammonium molybdate or 0.32 per cent inorganic phosphorus. After heating the solution one-half hour more 0.0010 gram  $\text{Mg}_2\text{P}_2\text{O}_7$  was obtained; further heating for one hour gave 0.0022 gram  $\text{Mg}_2\text{P}_2\text{O}_7$  and a fourth-hour-heating gave 0.0040 gram  $\text{Mg}_2\text{P}_2\text{O}_7$ . The total inorganic phosphorus obtained after heating three and one-half hours as above was 1.39 per cent. The results indicate that the cleavage under these conditions is slow and that it proceeds at a very uniform rate.

*Experiment to determine whether inosite is formed in the spontaneous decomposition of phytic acid.*

The sample of old phytic acid previously referred to was used. As shown by the analysis on p. 180 the preparation contained 3.10 per cent inorganic phosphorus. Of this acid, 12.8 grams, (corresponding to 10 grams of the dry substance) were dissolved in about 500 cc. of water and barium hydroxide (Kahlbaum alkali-free) added to slight alkaline reaction. The precipitate was filtered and washed several times in water. The barium precipitate was reserved for special examination.

The filtrate was examined for inosite as follows: The excess of barium hydroxide was precipitated with carbon dioxide, filtered and evaporated on the water bath nearly to dryness. The residue was taken up in a few cc. of hot water, filtered from a small amount of barium carbonate and the filtrate mixed with alcohol and ether and allowed to stand for several days in the ice chest. A trace of a white amorphous precipitate had separated but absolutely no inosite crystals appeared.

In case the organic part of the phytic acid molecule, corresponding to the inorganic phosphoric acid present, had separated as inosite the above quantity, 10 grams, should have contained about 0.3 gram of inosite and such a quantity could not have escaped detection. Since no inosite could be isolated it seems fair

to assume that under the above conditions of spontaneous decomposition phytic acid does not decompose into inosite and phosphoric acid but into phosphoric acid plus some unknown substance.

*Examination of the above barium precipitate.*

In the hope of throwing some light upon the nature of this unknown substance the barium precipitate obtained on the addition of barium hydroxide was examined as follows: It was rubbed up with about 400 cc. of 0.5 per cent hydrochloric acid and brought into solution by the careful addition of dilute hydrochloric acid. After filtering, it was precipitated by adding an equal volume of alcohol. The precipitate was filtered, washed in dilute alcohol, dissolved in 0.5 per cent hydrochloric acid and reprecipitated by barium hydroxide. The substance was then precipitated twice from 0.5 per cent hydrochloric acid with alcohol, finally filtered, washed in dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid. A white amorphous powder was obtained which weighed 14 grams. It was free from chlorides and inorganic phosphate. After drying at 105° in vacuum over phosphorus pentoxide the following results were obtained:

Found: C = 7.83; H = 1.46; P = 16.72; Ba = 36.96 per cent.

The carbon found is much too high for a pure barium phytate.

*Preparation of crystallized barium phytate from the above amorphous barium salt.*

The substance was rubbed up in a mortar with about 150 cc. of cold water and allowed to stand for several hours. The insoluble portion was changed slowly into a semi-crystalline precipitate. It was filtered and washed in water and then recrystallized as follows: It was dissolved in a small quantity of about 3 per cent hydrochloric acid, the free acid nearly neutralized with barium hydroxide; a concentrated solution of 10 grams of barium chloride was added, the solution filtered and alcohol added gradually with constant shaking until a slight permanent cloudiness was produced. On standing the substance crystallized slowly in the usual crystalline form, i.e., in globular masses of microscopic needles. After two days the crystals were filtered off, washed free of chlorides in water

and then in alcohol and ether and dried in the air. Yield, 4.5 grams. The substance gave no reaction with ammonium molybdate.

A further quantity of the same shaped crystals was obtained from the aqueous solution containing the water-soluble portion of the amorphous salt by adding to it 2.5 grams of barium chloride and allowing to stand over night. The balance of the water-soluble portion of the substance was recovered by precipitating with an equal volume of alcohol. The resulting precipitate was filtered, washed free of chlorides with dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid. Yield, 4.1 grams.

These substances were analyzed after drying at  $105^{\circ}$  in vacuum over phosphorus pentoxide. The recrystallized salt gave the following results:

Found: C = 6.28; H = 1.23; P = 15.93; Ba = 42.18;  $H_2O$  = 11.81 per cent.

The crystalline salt which separated from the aqueous solution gave:

C = 6.47; H = 1.23; P = 15.95; Ba = 42.77;  $H_2O$  = 12.62 per cent.

These substances are therefore nearly pure hepta-barium salts of inosite hexa-phosphate.

Calculated for  $(C_6H_{11}O_{24}P_6)_2Ba_7$  = 2267:

C = 6.35; H = 0.97; P = 16.40; Ba = 42.39 per cent.

The water-soluble substance precipitated with alcohol gave the following:

Found: C = 8.58; H = 1.62; P = 15.86; Ba = 38.28 per cent.

This substance was again treated with about 100 cc. of cold water, the insoluble portion filtered off and the filtrate, after adding 1 gram of barium chloride, precipitated with alcohol. After washing in dilute alcohol, alcohol and ether and drying in vacuum over sulphuric acid 1.4 grams of a white amorphous substance were obtained. For analysis it was dried at  $105^{\circ}$  in vacuum over phosphorus pentoxide.

Found: C = 8.08; H = 1.68; P = 15.64; Ba = 39.75 per cent.

This water-soluble substance apparently represents a mixture of the barium salts of penta- and tetra-phosphoric acid esters of inosite.

*Concerning the decomposition products of phytic acid after drying at 105° under reduced pressure.*

The specimen of old phytic acid previously examined was used. 12.8 grams (corresponding to 10 grams dry acid) were dried at 105° for about forty-eight hours over sulphuric acid under slightly reduced pressure. It was then dissolved in about 200 cc. of cold water. The solution was practically black in color and contained particles of carbonized material. It was decolorized by shaking with animal charcoal. The clear, colorless solution was then precipitated with barium hydroxide to slight alkaline reaction; the precipitate filtered and washed in water and reserved for examination. The filtrate and washings were freed from barium with carbon dioxide and evaporated on the water bath to dryness. The residue was taken up in a small amount of hot water and filtered. On adding a little alcohol a heavy voluminous white amorphous precipitate was produced. This was removed from the solution by adding about three volumes of alcohol. The precipitate settled, leaving a clear supernatant liquid; adding more alcohol produced no further precipitate. It was then filtered and washed in alcohol and the filtrate reserved.

After drying, the above precipitate was obtained as a heavy, white, amorphous powder. It was free from inorganic phosphorus but contained barium and after combustion the ash gave a heavy yellow precipitate with ammonium molybdate. This substance was purified as will be described later.

The filtrate from the above precipitate was again evaporated on the water bath nearly to dryness, taken up in hot water, filtered and mixed with alcohol and ether. On scratching with a glass rod a substance began to crystallize in needles. It was allowed to stand in the ice-chest over night. The crystals were then filtered, washed in alcohol and ether and dried in the air. Yield, 0.25 gram. The substance was recrystallized four times in the same manner and was finally obtained in colorless needles free from water of crystallization. It gave the reaction of Secherer and melted at 222° (uncorrected). It was, therefore, no doubt pure inosite. This was further confirmed by the analysis:

0.1215 gram substance gave 0.0737 gram  $H_2O$  and 0.1780 gram  $CO_2$ .

Found: C = 39.95; H = 6.78 per cent.

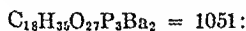
For  $C_6H_{12}O_6$  = 180: Calculated, C = 40.00; H = 6.66 per cent.

*Purification of the barium- and phosphorus-containing precipitate removed from the inosite solution with alcohol.*

The substance, mentioned above, precipitated with alcohol, was apparently the barium salt of an organic phosphoric acid but it differed in solubility from any other salt of this nature previously observed. It was very soluble in water and was not precipitated from the aqueous solution by barium hydroxide. The dry substance weighed 1.2 grams. It was dissolved in a small quantity of water, a few drops of dilute hydrochloric acid added and 10 cc. of  $\frac{1}{2}$  barium chloride. The solution was heated to boiling and alcohol added until a slight cloudiness was produced. On standing in the cold over night a small amount of a hard crust had separated on the bottom of the flask. This was removed and the solution again heated and more alcohol added when a further quantity separated in the same way. The substance was finally filtered and washed thoroughly in 80 per cent alcohol, alcohol and ether and dried in the air. Without further purification the substance was analyzed after drying at 105° in vacuum over phosphorus pentoxide.

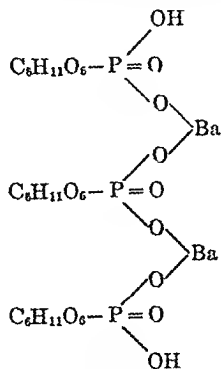
Found: C = 20.03; H = 3.58; P = 8.53; Ba = 25.55 per cent.

The small quantity precluded recrystallization and we are therefore unable to state whether the substance was pure. The analytical result indicates that it was a barium salt of inosite monophosphate and agrees approximately with this formula:



Calculated, C = 20.55; H = 3.33; P = 8.84; Ba = 26.16 per cent.

Such a salt could be represented by the following formula:



*Examination of the precipitate produced with barium hydroxide after drying the above acid.*

A portion of the barium preeipitate was dried in vacuum over sulphuric acid and then examined for total and inorganic phosphorus in the same way as before.

Found: Total phosphorus (by Neumann method), 9.98 per cent.

Found: Inorganic phosphorus, 7.46 per cent.

As will be noticed from these figures 74.76 per cent of the phosphorus was present as inorganic phosphoric acid.

The substance was freed from inorganic phosphate by precipitating four times with alcohol from 0.5 per cent hydrochloric acid. After finally drying in vacuum over sulphuric acid 3.2 grams of a snow-white, amorphous powder were obtained. The substance was free from ehlorides and inorganic phosphate.

It was shaken up with about 75 cc. of cold water in which the greater portion dissolved; 10 cc. of  $\frac{1}{2}$  barium chloride were added and allowed to stand for several hours; the insoluble portion was then filtered off, washed free of chlorides with water and then in alcohol and ether and dried in vacuum over sulphuric acid. It weighed 0.65 gram.

The filtrate from above containing the water-soluble portion of the substance was acidified with a few drops of dilute hydrochloric acid, heated to boiling and alcohol added until a slight permanent cloudiness remained. On standing over night, a portion had separated in the form of a heavy granular powder. Under the microscope no definite crystal-form could be observed but it appeared to consist of transparent globules. It was filtered off, washed free of ehlorides in 30 per cent alcohol, alcohol and ether and dried in the air. Yield, 0.67 grams. It was free from inorganic phosphate.

The mother-liquor from above was precipitated with alcohol. After settling, the precipitate was filtered, washed with dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid. Yield 1.55 grams. The substance was a snow-white, amorphous powder. It was free from chlorides and inorganic phosphate.

These three different portions were analyzed after drying at  $105^{\circ}$  in vacuum over phosphorus pentoxide.

The water-insoluble portion gave:

C = 9.40; H = 1.65; P = 13.76; Ba = 39.56 per cent.

Judging by the analysis, this substance consists mainly of the barium salt of inosite tetraphosphate.

The granular powder which separated from the hot dilute hydrochloric acid solution and alcohol on cooling gave the following result:

C = 12.70; H = 2.40; P = 13.84; Ba = 32.29; H<sub>2</sub>O = 12.77 per cent.

This substance appears to be mainly the barium salt of inosite triphosphate, although not pure. It was mixed probably with some barium salt of inosite diphosphate.

The water-soluble portion precipitated with alcohol gave:

C = 14.07; H = 2.31; P = 12.92; Ba = 33.04 per cent.

Deducting the barium found, allowing for hydrogen and water, and calculating to the free acid, these results became: C = 20.88; H = 4.11; P = 19.16 per cent.

This is approximately the composition of inosite diphosphate, C<sub>6</sub>H<sub>11</sub>O<sub>12</sub>P<sub>2</sub> = 340; Calculated, C = 21.17; H = 4.11; P = 18.23 per cent.

That these substances, separated from the partially decomposed phytic acid, are inosite esters of phosphoric acid and not condensation or other decomposition products is evident from the fact that on complete cleavage inosite is obtained. Unfortunately the amount of each of the above substances was too small to permit of examination in this direction except the last one, viz., the water-soluble product precipitated by alcohol and which analyzed for inosite diphosphate. The remainder (0.94 gram dry substance) was hydrolyzed with dilute sulphuric acid in a sealed tube at 150°-160° for about two and one-half hours and the inosite isolated in the usual way. The amount of inosite obtained was 0.28 gram or about 88 per cent of the theory. The substance gave the reaction of Scherer and melted at 222° which leaves no doubt that it was pure inosite.

It is evident that all of the barium precipitates described above are mixtures. It could hardly be expected that a complete separation into pure chemical compounds of the salts of these inosite esters could be effected by the method used.

The analytical results, however, show that it is possible to isolate from partially decomposed phytic acid certain substances approximating in composition various phosphoric acid esters of inositol which on complete cleavage yield inositol just as phytic acid itself. This fact, we believe, supports the view previously expressed that phytic acid suffers a gradual and partial decomposition, *i.e.*, molecules of phosphoric acid are eliminated one by one. We believe also that these facts taken in connection with the formation of inositol from phytic acid on mere drying at  $105^{\circ}$  must be considered as a strong support of the theory that phytic acid is inositol hexaphosphate and not some complex compound as previously held.

*Attempt to prepare methyl ester of phytic acid.*

The silver salt previously described as hepta silver phytate<sup>12</sup> was used. 5.4 grams of this salt were suspended in 100 cc. of absolute methyl alcohol and 4 grams of methyl iodide (a little over the required amount) were added and the mixture shaken for several hours, the flask being protected from the light. At the end of this time the white silver phytate had changed into the yellow silver iodide. The precipitate was filtered off and washed several times in absolute methyl alcohol and the filtrate evaporated in vacuum several times to dryness under addition of methyl alcohol for the removal of the excess of methyl iodide. The residue was dissolved in methyl alcohol and evaporated to dryness in vacuum over sulphuric acid. The substance was then obtained as a light yellow colored, thick syrup of faint aromatic odor. It was strongly acid in reaction and of sharp acid taste. For analysis it was dried in vacuum at  $105^{\circ}$  over phosphorus pentoxide. It then turned very dark in color.

0.1985 gram substance gave 0.0608 gram  $H_2O$  and 0.1016 gram  $CO_2$ .

Found: C = 13.95; H = 3.42 per cent.

This agrees with a dimethyl ester of phytic acid. For  $C_6H_{16}O_8P_6(CH_3)_2$  = 688. Calculated, C = 13.95; H = 3.19 per cent.

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<sup>12</sup> This *Journal*, xii, p. 107, 1912; Tech. Bulletin 21, N. Y. Agric. Exp. Station, 1912.

*The water-insoluble substance which separates from phytic acid after drying.*

As has been mentioned earlier, phytic acid, which has been dried to constant weight in vacuum over phosphorus pentoxide, is not completely soluble in water. We have observed this insoluble substance in many instances after drying phytic acid at 60°, at 78° and at 105°. It always separates on adding water to the dry substance in thin gelatinous plates. It appears to be practically insoluble in hot or cold water. Continued boiling in acidulated water is necessary to dissolve it. It is also insoluble in alcohol and ether.

In order to obtain some knowledge of the composition of this insoluble substance 2.7 grams of phytic acid, containing about 12 per cent of moisture, were dried to constant weight at 105° in vacuum over phosphorus pentoxide. After treating with water the insoluble portion was filtered, washed thoroughly in water and finally in alcohol and ether and dried in vacuum over sulphuric acid. It was then obtained as a dirty gray powder which weighed 0.23 gram. It was non-hygroscopic. For analysis it was dried at 105° in vacuum over phosphorus pentoxide at which no change in color was noticeable. The substance was burned with copper oxide and the phosphorus determined in the ash.

0.2118 gram dry substance gave 0.0569 gram  $\text{H}_2\text{O}$ , 0.1357 gram  $\text{CO}_2$  and 0.1822 gram  $\text{Mg}_2\text{P}_2\text{O}_7$ .

Found: C = 17.47; H = 3.00; P = 23.98 per cent.

The quantity of the substance obtained was so small that it was only sufficient for one analysis. Of course, we are unable to state whether it was homogeneous or not but the analytical results agree approximately with inosite triphosphate minus one molecule of water. The substance may, therefore, be a partial pyrophosphoric acid ester of inosite or it may represent some complex decomposition product of phytic acid.

In conclusion we present a summary of the analytical results of the preceding crystalline barium salts in comparison with the calculated percentages required for the usual phytic acid formula and inosite hexaphosphate.

TABLE I.

*Barium salts crystallized from dilute hydrochloric acid by the addition of alcohol.*

	FROM COT- TONSEED MEAL: FOUND	FROM OATS: FOUND	FROM CORN: FOUND	FROM COM- MERCIAL PHYTIN: FOUND	CALCULATED FOR TRI-BARIUM INOSITE HEXAPHOS- PHATE $C_6H_{11}O_{21}P_6Ba_3$	CALCULATED FOR TRI-BARIUM PHYTATE $C_6H_{11}O_{21}P_6Ba_3$
	per cent	per cent	per cent	per cent	per cent	per cent
C.....	6.61, 6.59	6.50	6.50	6.64	6.75	6.42
H.....	1.34, 1.44	1.56	1.40	1.39	1.12	1.60
P.....	16.91, 17.08	17.00	17.08	17.15	17.44	16.60
Ba.....	39.57, 38.79	38.01	39.14	38.62	38.65	36.78

TABLE II.

*Barium salts crystallized from dilute hydrochloric acid in the presence of barium chloride.*

	COTTON SEED MEAL: FOUND	OATS: FOUND	CORN: FOUND	COMMER- CIAL PHYTIN: FOUND	HEPTA-BARIUM INOSITE HEXA- PHOSPHATE $(C_6H_{11}O_{21}P_6)_2$ Ba <sub>7</sub>	HEPTA-BARIUM PHYTATE $(C_6H_{11}O_{21}P_6)_2$ Ba <sub>7</sub>
	per cent	per cent	per cent	per cent	per cent	per cent
C.....	6.29, 6.03	6.23	6.27	6.27	6.35	6.06
H.....	1.11, 1.18	1.27	1.32	1.19	0.97	1.43
P.....	16.54, 15.80	16.17	16.28	16.22	16.40	15.66
Ba.....	42.06, 42.85	41.48	42.06	42.03	42.39	40.46

TABLE III.

*The free acids prepared from the crystalline barium salts.*

	COTTON- SEED MEAL: FOUND	OATS: FOUND	CORN: FOUND	COMMER- CIAL PHYTIN: FOUND	INOSITE HEXA- PHOSPHATE $C_6H_{11}O_{21}P_6$	PHYTIC ACID ACCORDING TO NEUBERG. $C_6H_{11}O_{21}P_6$
	per cent	per cent	per cent	per cent	per cent	per cent
C.....	10.68	10.82	10.86	10.80	10.90	10.08
H.....	3.09	3.09	3.02	3.23	2.72	3.36
P.....	27.66	27.12	27.22	27.31	28.18	26.05

The author desires to express his appreciation and thanks to Dr. P. A. Levene of the Rockefeller Institute for Medical Research, New York, N. Y., and to Dr. Thomas B. Osborne of the Connecticut Agricultural Experiment Station, New Haven, Conn., for many suggestions which have been of great value in carrying out the work reported in this and the three preceding papers.

# CAROTIN—THE PRINCIPAL NATURAL YELLOW PIGMENT OF MILK FAT: ITS RELATIONS TO PLANT CAROTIN AND THE CAROTIN OF THE BODY FAT, CORPUS LUTEUM AND BLOOD SERUM.<sup>1</sup>

## I. THE CHEMICAL AND PHYSIOLOGICAL RELATION OF THE PIGMENTS OF MILK FAT TO THE CAROTIN AND XANTHOPHYLLS OF GREEN PLANTS.

By LEROY S. PALMER AND C. H. ECKLES.

(From the Dairy Chemistry Laboratory, University of Missouri, in coöperation with the U. S. Department of Agriculture.)

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For a number of years the great variety of yellow animal pigments have been classified under the general name lipochrome. Recent investigations have shown, however, that some of these pigments are in reality very closely related chemically or are identical with the carotin or xanthophyll pigments of plants. Willstätter and Escher<sup>2</sup> have analyzed the pure lutein of egg yolk and found it to be isomeric with the crystalline xanthophyll of green plants; and Escher<sup>3</sup> has identified in the same manner the lipochrome of the corpus luteum as a true carotin. The isolation of the pigments was in both cases attended with great difficulty. Only 4 grams of crude crystalline pigment were obtained from 6000 hen eggs, and less than 0.5 gram of crystalline carotin from 10,000 cows' ovaries.

The natural yellow pigment of butter is the most commonly observed of all animal lipochromes. It is also more important from a commercial standpoint than any other animal pigment. The public judges the richness of dairy products by their yellow color, and demands that butter especially shall have a standard shade of yellow. The pigment of butter fat, however, has been the least

<sup>1</sup> Published by permission of the Secretary of Agriculture.

<sup>2</sup> *Zeitschr. f. physiol. Chem.*, lxxvi, pp. 214-225, 1912.

<sup>3</sup> *Ibid.*, lxxxiii, p. 198, 1913.

investigated of all animal lipochromes. Thudichum's<sup>4</sup> classical investigation included the pigment of butter fat under the general classification lutein, which he proposed. No other study of the butter-fat pigment has been reported in the literature. The pigment is usually classified in the current text-books<sup>5</sup> according to Krukenberg's classification of lipochromes.

The object of the investigation here reported was to classify the butter-fat pigment not only as a true lipochrome, but also with respect to its relations to the carotin and xanthophylls of green plants. In addition we have gathered considerable information relative to the influence of certain factors upon the color of butter, among which may be mentioned the character of the ration and the breed of the cow.

#### EXPERIMENTAL.

##### *Isolation of the butter-fat pigment.*

The statement is frequently met in the literature<sup>6</sup> and in text-books and works<sup>7</sup> on oils and fats, that the pigment of butter or butter fat appears in the unsaponifiable extracts along with cholesterol and other substances. The method of obtaining the crude pigment was therefore dependent upon obtaining the unsaponifiable matter in as high degree of purity<sup>8</sup> as possible. The method which we used in the following studies was as follows: The butter fat was saponified with a 20 per cent solution of alcoholic potash using 2 cc. per gram of fat. Saponification was allowed to continue for one-half to one hour at the temperature of the boiling solution. The resulting soap was dissolved in three volumes of distilled water. After cooling, this solution was shaken with an equal volume of pure ether<sup>9</sup> in a separatory funnel. The extraction

<sup>4</sup> *Proc. Roy. Soc.*, xvii, p. 253, 1869.

<sup>5</sup> Such as Hammarsten: *Text-Book of Physiological Chemistry*.

<sup>6</sup> Such as Kirsten: *Zeitschr. Nahrungs- u. Genussm.*, v, p. 833, 1903.

<sup>7</sup> Lewkowitsch: *Oils, Fats and Waxes*, Vol. 1, p. 371 (1909 edition).

<sup>8</sup> The chief thing to be avoided here is the formation of aldehyde resin pigments. The alcohol must be freed from impurities causing these pigments by distillation over KOH, and strong potash solutions avoided in the saponification of the fat.

<sup>9</sup> Kahlbaum's reagent distilled over sodium was used throughout these studies.

was repeated with a fresh volume of ether equal to one-half the volume of the soap solution. The soap was now colorless. The combined ether extracts were washed many times with an excess of water, carefully at first to avoid emulsions, and more vigorously with subsequent washings. When the wash water no longer reacted alkaline to phenolphthalein, the golden yellow ether solution was either dried over fused  $\text{CaCl}_2$ , or let stand several hours to allow the excess moisture to settle out. In either case the ether was carefully decanted and evaporated to dryness at a low temperature, leaving a salve-like residue of various tints of yellow to red, depending upon the amount of fat used and the depth of its color.

This residue was in many cases dissolved in warm 95 per cent alcohol and freed from cholesterol by the digitonin method of Windaus.<sup>10</sup> No pigment was carried down by the digitonin-cholesteride, which could be filtered off, leaving the pigment in solution. The impurities which remained, traces of fat and lecithin decomposition products, did not interfere with the study of the pigment. In fact, it was found that the cholesterol was not a serious hindrance to the study of the properties of the pigment.

#### *Methods of identifying butter-fat pigments.*

The nature of the substances with which the pigment of butter fat is associated and their very great relative proportion, especially the fat, at once precluded the isolation of the pigment in sufficient quantity to establish its chemical composition and molecular weight. We were therefore forced to confine ourselves to a macroscopic identification. Fortunately the characteristics and properties of the carotin and xanthophylls of plants are so well established that their demonstration is at present a matter of no great difficulty to any one familiar with the properties of these pigments.

Preliminary to the study of the butter-fat pigment, a careful study was made of the carotin and xanthophyll pigments which we isolated by extracting green alfalfa hay<sup>11</sup> with carbon bisulphide. Among the many properties of the two classes of pigments, we selected the following as being the most characteristic and applicable to an unknown pigment: 1. The solubility properties. 2.

<sup>10</sup> Windaus: *Zeitschr. f. physiol. Chem.*, lxxv, p. 110, 1909.

<sup>11</sup> The dried powdered leaves of the hay were used.

The adsorption properties with respect to  $\text{CaCO}_3$ . 3. The spectroscopic absorption properties, particularly in carbon bisulphide solution.

What we have called the "solubility properties" are the great difference in relative solubility which the carotin and xanthophylls show toward the alcohols and petroleum ether or towards the alcohols and carbon bisulphide. These have been thoroughly investigated by Tswett<sup>12</sup> and by Willstätter and Mieg.<sup>13</sup> It has been found that carotin cannot be extracted from its solution in low-boiling-point petroleum ether by 80-90 per cent alcohol (either ethyl or methyl) while low-boiling-point petroleum ether will quantitatively extract carotin from its solution in 80-90 per cent alcohol. When the two solvents used are alcohol and carbon bisulphide, the latter acts like the petroleum ether. The xanthophylls on the other hand show exactly the reverse action. Petroleum ether will not extract them from their solution in 80-90 per cent alcohol, while the latter solvent will quantitatively extract them from their solution in petroleum ether. The relative solvent power of alcohol and carbon bisulphide toward xanthophylls is not so clearly defined, these two pigments being about equally soluble in the two solvents.

The application of these properties is seen in the separation of the carotin from xanthophylls or vice versa, depending upon whether the mixed pigments are in the carotin solvent, *i.e.*, the petroleum ether, or in the xanthophyll solvent, *i.e.*, 80-90 per cent alcohol. For example, if a petroleum ether solution of xanthophylls and carotin is shaken with 80-90 per cent alcohol the xanthophylls will be extracted, leaving the carotin in the upper petroleum ether layer. This separation can be made very nearly quantitative, if each solution thus obtained is shaken with a fresh amount of the other solvent and this procedure continued until no more color is extracted from the petroleum ether by fresh alcohol, and until no more color is extracted from the alcohol by fresh petroleum ether.

The adsorption properties of carotin and xanthophylls used throughout these experiments were an application of Tswett's<sup>14</sup>

<sup>12</sup> *Ber. d. d. bot. Gesellsch.*, xxiv, pp. 316, 384, 1906; xxix, p. 630, 1911.

<sup>13</sup> *Ann. d. Chem.*, ccciv, p. 1, 1907.

<sup>14</sup> *Loc. cit.*

interesting discovery that carotin when in anhydrous carbon bisulphide (or petroleum ether) solution is not adsorbed by pure, dry  $\text{CaCO}_3$ , while xanthophylls, under the same conditions, are adsorbed to a greater or less degree. Tswett has made a beautiful application of this discovery to a solution of carotin, xanthophylls and chlorophyll pigments, whereby he separated the pigments from one another by filtering the solution of mixed pigments through a column of dry  $\text{CaCO}_3$ , moistened first with the solvent being used ( $\text{CS}_2$  or petroleum ether). The result was that as the pigments passed through the column with the aid of suction and a stream of the pure solvent, they differentiated themselves into various zones, depending upon the adsorption affinity of the  $\text{CaCO}_3$  for each pigment. Carotin, being unadsorbed by the  $\text{CaCO}_3$  passed through first as a rose or orange colored ring or zone, and could be collected by itself at the mouth of the glass tube, which is drawn out somewhat at the end. The arrangement for performing such a chromatographic analysis is shown in figure 1. The details of such an analysis may be found by referring to Tswett's work.

As stated above, use was also made of the difference in the position of the spectroscopic absorption bands of carotin and xanthophylls. It is now well known that the xanthophyll bands are shifted an appreciable amount towards the blue end of the spectrum from the corresponding bands of carotin. The means of accurately measuring the position of the absorption bands of the carotin and xanthophyll pigments were not available for this study. We had a spectroscope of narrow dispersion, which showed the absorption bands very clearly. It was equipped with an arbitrary scale. By setting this scale at a constant point before making any measurements, we were able to standardize the absorption bands of the plant carotin and xanthophylls so that the bands of unknown pigments could be compared with them. Such a standardization was made for the carotin and xanthophylls which were separated

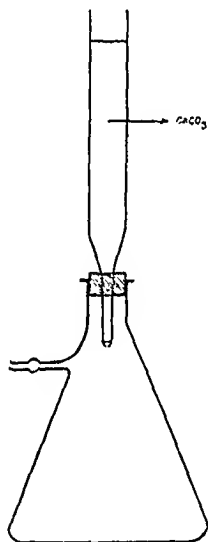


FIG. 1.

from green alfalfa hay and from the carrot by means of their solubility and adsorption properties described above. The solvents used for the spectroscopic studies were carbon bisulphide and ethyl alcohol. In the case of the former the color of the solution giving the bands was measured by the Lovibond tintometer. This was the only means available of standardizing the strength of the solution, which is an important factor, influencing the position of the bands, as is well known. All unknown solutions were adjusted to this color as nearly as possible before measuring the absorption bands. The spectroscopic standards for carotin and xanthophylls used throughout this study are given in Table 1.

TABLE 1.

*Spectroscopic standard of carotin and xanthophylls.*

(From the carrot.)

PIGMENT	LAYER	COLOR			ABSORPTION BANDS		
		Yellow	Red	Light	Band I	Band II	Band III
<i>In CS<sub>2</sub>.</i>							
	<i>mm.</i>						
Carotin.....	10	26.0	5.5	1.0	225-242	261-278	301-319
Xanthophyll.....	10	17.0	4.4	0.5	233-253	272-291	312-330
<i>In C<sub>2</sub>H<sub>5</sub>OH.</i>							
Carotin.....	10				257-275	303-318	345-354
Xanthophyll.....	10				263-280	305-325	355- —

It will be noticed that carotin as well as the xanthophylls showed three absorption bands.<sup>15</sup> This is contrary to the observations of Willstätter and Mieg.<sup>16</sup> The third band was very clear in all our carotin solutions, especially when in CS<sub>2</sub>, due to the shifting of all the bands toward the red when in this solvent. Since the pigment was free from any pigments adsorbed by CaCO<sub>3</sub> or extracted from low-boiling-point petroleum ether by 80-90 per cent alcohol, we are forced to conclude that it was not contaminated with xan-

<sup>15</sup> We found the absorption bands as well as the other properties of carotin and xanthophylls in no wise altered by saponification.

<sup>16</sup> *Loc. cit.*

thophylls. Other investigators, such as F. G. Kohl,<sup>17</sup> and C. A. Schunck<sup>18</sup> in his well-known spectro-photographic investigations, have observed three carotin absorptions bands. We are inclined to believe that the end absorption which Willstätter and his co-workers have observed for their crystalline carotin is in reality a third well defined absorption band.

An application of the above characteristic properties of carotin and xanthophylls to the unknown pigments throughout the studies reported in this paper as well as in the several papers which are to follow, consisted in the following procedure. The freshly prepared pigment, freed as far as possible from substances interfering with the above properties, such as fat and large quantities of cholesterol, was in some cases immediately subjected to a chromatographic analysis in pure carbon bisulphide solution (Merck's c.p. reagent). Any pigments thus differentiated as belonging either to the carotin or xanthophyll groups, were further examined in regard to their solubility properties<sup>19</sup> and spectroscopic absorption bands. In other cases the relative solubility analysis preceded the other analyses, *i.e.*, any pigments thus differentiated were analyzed chromatographically to determine their adsorption properties. This method usually resulted in a clear differentiation of all xanthophylls. Spectroscopic properties of all pigments thus separated were then studied in order to confirm the other properties.

#### *Identification of the pigment of butter-fat.*

Applying the above carotin and xanthophyll properties to the pigment isolated from a large number of samples<sup>20</sup> of butter fat, we found it to be composed of a mixture of carotin and xanthophylls, with the former pigment comprising by far the largest proportion of the entire pigment. About 30 grams of pure rendered fat were used for each study. In this way the isolation of the pigment and its entire study could be carried out very quickly. We found this to be essential to an accurate study of the character of

<sup>17</sup> *Untersuchungen über d. Karotin*, Leipzig, 1902.

<sup>18</sup> *Proc. Roy. Soc.*, lxxii, p. 170, 1903.

<sup>19</sup> Kahlbaum's petroleum ether (b.p. 30-50°C.) was used in all such studies, throughout this investigation.

<sup>20</sup> These samples varied in color from a very light to a deep yellow, including fat produced when carrots were fed.

the pigment on account of the ease with which the pigment is oxidized, and its characteristic properties rendered nil. This was found to be especially true of the spectroscopic absorption bands, which apparently disappear long before the pigment shows signs of bleaching.

As indicated above, it was found possible to separate from the main butter-fat pigment a small proportion of xanthophyll pigments. The amount of these pigments varied somewhat with different butter fats as did also the number of xanthophyll constituents. In one case the pigment which was extracted by 80-90 per cent alcohol from the petroleum ether solution of the mixed pigment of a high-colored butter fat from a Jersey cow, showed two and possibly three yellow adsorption zones when the  $\text{CS}_2$  solution was filtered through a column of  $\text{CaCO}_3$  in the manner previously

TABLE 2.

*Absorption bands of butter-fat pigments.*

BAND NO.	CAROTIN		XANTHOPHYLLS	
	In $\text{CS}_2$	In $\text{C}_2\text{H}_5\text{OH}$	In $\text{CS}_2$	In $\text{C}_2\text{H}_5\text{OH}$
1	224-242	256-274	232-249	264-297
2	259-278	298-312	272-291	306-326
3	302-319	345- —	312-330	356- —

described. The pigment in each of these zones showed the xanthophyll absorption bands.

The principal butter-fat pigment in every case was carotin. It could not be extracted from its solution in petroleum ether (b.p.  $30^\circ$ - $50^\circ\text{C}.$ ) by 80-90 per cent alcohol. Its carbon bisulphide solution had a blood-red or a deep red orange color. In this solvent it was not adsorbed by  $\text{CaCO}_3$  but on the other hand when filtered through a column of this material it passed rapidly through as a rose-colored zone, in every way identical with the carotin from green alfalfa hay and from the carrot. In carbon bisulphide as well as in alcohol, this pigment showed the identical absorption bands of vegetable carotin. The positions of the absorption bands of the carotin and xanthophylls from butter fat, measured according to the scale described above, are given in Table 2.

*The pigments of colostrum milk fat.*

Among the various samples of butter fat whose pigments were examined were several samples of the very high-colored fat of colostrum milk. The very deep yellow color of the colostrum milk is a familiar phenomenon. It does not seem to be generally recognized, however, that this high color is usually<sup>21</sup> due entirely to the high color of the fat of this milk. We have observed many times that when the fat is entirely removed from colostrum milk, the skim milk has the appearance of ordinary skim milk, and the butter and

TABLE 3.  
*Color of the fat of colostrum milk.*

COW NO.	BREED	ROUGHAGE FED	DAYS AFTER PARTURITION	COLOR		
				Yellow	Red	Light
301	Ayrshire	Alfalfa*	4	78	3.5	1.0
301	Ayrshire	Alfalfa	26	71	1.5	0.5
300	Ayrshire	Alfalfa	4	71	3.5	1.0
300	Ayrshire	Alfalfa	20	68	2.8	1.0
2	Jersey	Alfalfa	13	68	2.6	0.5
2	Jersey	Alfalfa	22	57	2.5	0.5
2	Jersey	Alfalfa	2	54	4.3	1.0
2†	Jersey	Alfalfa	20	50	2.5	1.0
20	Jersey	Alfalfa	2	47	4.8	1.0
20	Jersey	Alfalfa	25	47	2.0	0.2
206	Holstein	Alfalfa	1	50	4.7	0.3
206	Holstein	Alfalfa	5	54	2.0	0.2

\*The alfalfa hay was rich in carotin and xanthophylls.

†Second sample taken after next parturition.

the rendered fat have a depth of color which is never equaled at any subsequent stage of the lactation period. This characteristic of colostrum milk is common to all breeds of cows, and the high color continues in cows of all breeds for a short time after parturition and then gradually falls off. Table 3 gives the color of the milk fat of several cows shortly after parturition and again a week or two later. The color readings are the Lovibond tintometer readings on a 1-inch layer of melted, rendered fat.

<sup>21</sup> Colostrum milk is occasionally contaminated with blood.

An examination of the character of the pigments of colostrum milk fat showed that they are composed of the same pigments found in normal milk fat. The high color of colostrum milk is therefore due merely to a high concentration of carotin and xanthophylls, especially carotin. Some study of the cause of this phenomenon will be reported in a later paper.

*The physiological relation between the pigments of milk fat and the carotin and xanthophylls of plants.*

General observation for no doubt hundreds of years, at least ever since butter has become of importance in the diet of man, has shown that green feeds of all kinds, especially fresh green grass, greatly increase the color of butter fat. Other feeds, such as carrots, beets and yellow corn have been said to have the same effect. On the other hand it is said that the color of butter is decreased by some feeds, among which may be mentioned cottonseed meal.

The establishment of the identity of the pigment of butter fat with the carotin and xanthophylls of plant origin, at once offers an explanation of this variation in the color of butter with different feeds. It is readily seen that it is only the feeds which are rich in carotin and xanthophylls which are said to increase the color of butter fat. It would be inferred that cottonseed meal, which is said to decrease the color of butter, is devoid of these pigments.

In order to experimentally establish such a physiological relation between the pigment of milk fat and the carotin and xanthophylls of plants, it first became necessary to study the character of the pigments of some of the common cattle feeds. Among the roughages, the feeds studied were green alfalfa hay, bleached clover and timothy hay, corn silage, and cottonseed hulls. Among the concentrates, corn, both yellow and white, bran, cottonseed and linseed meals and other common feeds were studied. In addition, a careful study was made of the pigments of the carrot.

The results of these studies<sup>22</sup> showed that all the feeds, with the exception of the white corn, contained some carotin and xanthophylls. With the exception of green alfalfa hay, green grass,

<sup>22</sup> A detailed account of these studies may be found in Research Bulletin No. 10, Missouri Agricultural Experiment Station.

carrots and yellow corn, the amount of these pigments was negligible, in comparison with the amount in the feeds mentioned. The carotin and xanthophylls of cottonseed meal and hulls was found in the oil which these feeds contain. Carrots were found to contain considerable xanthophyll,<sup>23</sup> no less than eight xanthophyll constituents being separated by an adsorption chromatographic analysis. The principal pigment of the carrot, however, was carotin. It was surprising to find that the pigment of yellow corn was largely a xanthophyll. At least the characteristics of the pigment were more nearly those of a xanthophyll than of a carotin, although in general its properties were less well defined than those of any of the pigments obtained from any of the other foods studied. The character of the pigment of yellow corn has an important bearing on the results of the feeding experiments reported below.

### *The feeding experiments.*

By making use of the preceding experiments, the feeds were divided into two groups, one which would furnish an abundance of carotin and xanthophylls, and the other the least possible amount. A number of feeding experiments were then carried out to show the relation between these feeds and the color of butter fat. In order that the results might be comparative, the color of the fat in each case was determined in 1-inch layer, when in the liquid state, using the Lovibond tintometer. Some idea of the significance of the Lovibond color units may be obtained by stating that rendered "June" butter in 1-inch layer will give a color of 80 to 60 units of yellow. Color readings between 45 and 25 units of yellow would accordingly indicate fairly well colored to light colored butter; between 20 and 8 units of yellow would be called light to very light colored butter; below 8 ranging down to 1 or 2 units of yellow would be called white to "dead" white, especially if the fat was still in the form of butter.

*Experiment 1.* The ration of Cow No. 57, a pure bred Jersey, was changed from green alfalfa hay and yellow corn to bleached clover hay and white corn and the effect on the color of the butter

<sup>23</sup> The presence of a crystalline xanthophyll in the carrot has been observed by Euler and Nordenson: *Zeitschr. f. physiol. Chem.*, lvi, p. 223 1908.

and cottonseed meal. After about three weeks on this ration, the entire grain was changed to yellow corn. This was finally increased to 12 pounds per day. The effect on the color of the milk fat is shown in Table 6.

TABLE 6.

*The effect of a non-pigmented ration and a ration containing yellow corn upon the color of milk fat. (Ayrshire Cow No. 301.)*

DATE OF SAMPLE	ALFALFA HAY	CORN	COTTONSEED MEAL	COLOR OF BUTTER FAT	
				Yellow	Red
1912	lbs.	lbs.	lbs.		
October 3.....	16	6*	2	27.0	1.7
October 24.....	16	8†		7.5	1.2
November 1... ..	16	12		9.5	1.2
November 4.....	16	12		8.0	1.2

\* October 3-21, white corn.

† October 24-November 4, yellow corn.

The result was in perfect accord with the previous experiment showing that yellow corn is not a source of pigment for the milk fat of dairy cows.

*Experiment 4.* This experiment was conducted with the same cow as the preceding experiment and immediately followed that experiment. The purpose of the experiment was to test the supposition that a long continued feeding of a ration practically free from carotin and xanthophylls would ultimately result in the elimination of the normal storage of pigment in the body and the

TABLE 7.

*The effect of a long continued feeding of a non-pigmented ration upon the color of milk fat. (Cow No. 301.)*

DATE OF SAMPLE	ALFALFA HAY	COTTON-SEED HULLS	COTTON-SEED MEAL	CORN	COLOR OF BUTTER FAT	
					Yellow	Red
1912-13	lbs.	lbs.	lbs.	lbs.		
November 5.....	6.5			12	9.0	1.2
November 15.....	2.0	6	6		5.0	0.9
November 25.....		16	8		3.0	0.7
January 7.....		16	8		1.3	0.4

production of practically colorless milk fat. The ration used for this purpose was cottonseed meal and cottonseed hulls. The results are summarized in Table 7.

The above-stated supposition was fully borne out by the experiment. At the end of fifty-two days the cow was producing absolutely colorless butter. Only when the rendered fat was viewed in the tintometer could a very slight amount of color be detected. This might have been due to one or more of several factors; the blood-serum storage was not completely exhausted of pigment; the body fat may have been supplying a little; and a very small amount may have come from the oil in the ration.

*Experiment 6.* The color of the milk fat of Cow 301 was now so low that the conditions were considered ideal for two additional experiments, (1) a confirmation of the negative effect of yellow corn, (2) a study of carrot feeding, where the pigment fed would be almost pure carotin. About the time this experiment was begun it was found necessary to replace part of the cottonseed hulls with timothy hay which was not quite free from carotin. The results were fortunately not vitiated in any way by this change, as there was no apparent effect on the color of the milk fat. The results of the two experiments are given in Table 8.

The results of the first part of this experiment show conclusively that yellow corn plays no part in the pigmentation of milk fat. No reason has yet been given for this apparent anomaly. It unquestionably lies in the fact, as shown earlier in this paper, that the pigment of yellow corn is to be classed as a xanthophyll. This class of pigments apparently plays but a small part in the pigmentation of milk fat.

One or two points in connection with the carrot feeding need explanation. In the first place, neither the rise in color of the fat nor its rapidity after the addition of the carrots were commensurate with the amount of carrots fed. Apparently however, this was due to some physiological disturbance, for the animal eventually went off feed, making it necessary to withdraw her entire ration for a day or two as indicated in the table. When the animal had recovered, and a smaller amount of carrots fed, *i.e.*, 20 pounds per day against 50 pounds fed previously, the rise in color was very rapid, and soon reached a maximum for the ration. It remained in this neighborhood until the carrots were withdrawn, when the color slowly dropped again.

During the second part of the carrot feeding, the question arose as to whether a moderate coloration only of the milk fat was obtained when feeding 20 pounds of carrots per day because insufficient carotin was fed to give a higher colored fat. An examination of the feces showed, however, that carotin was being abundantly excreted, so the animal was apparently absorbing all the carotin it could.

TABLE 8.

*The effect of a ration containing yellow corn and of a ration containing carrots upon the color of milk fat. (Ayrshire Cow No. 301.)<sup>25</sup>*

DATE OF SAMPLE	ROUGH-AGE*	CARROTS	COTTON-SEED MEAL	CORN	COLOR OF BUTTER FAT	
					Yellow	Red
1912	lbs.	lbs.	lbs.	lbs.		
January 3.....	14		4	4 white	1.2	0.4
January 28.....	12		2	6 yellow		
February 6.....	12		2	6 yellow	2.0	0.5
February 7.....	12	6	4	4 white	2.2	0.5
February 8.....	12	20	4	4 white	2.2	0.5
February 9.....	12	30	4	4 white	2.3	0.5
February 18.....		20			19.0	1.1
February 19.....	3				15.0	1.1
February 20.....		5			10.0	1.3
February 21.....	3	10	1	1 white	8.0	1.2
February 22.....	8	20	2	2 white	9.0	1.3
February 26.....	12	20	4	4 white	36.0	1.8
March 6.....	12	50	4	4 white	24.0	1.3
March 7.....	12		4	4 white	23.0	1.3
March 30.....	12		4	4 white	7.5	1.3

\* Roughage consisted of two parts bleached timothy hay and one part cottonseed hulls.

This was confirmed by an examination of the blood, which showed the maximum coloration which is obtained under any conditions.<sup>26</sup> It must be concluded therefore that other factors, beside an excess

<sup>25</sup> During the period from February 18, p.m., to February 23, a.m., the cow was badly off feed and her entire ration was withdrawn for a few days. She soon recovered, however, and was put back on the experimental ration, not containing so many carrots, however.

<sup>26</sup> It will be shown in a later paper that the blood is capable of taking up only a definite maximum amount of carotin, which is independent of any factor save an excess of carotin in the food.

of carotin in the food, play a part in the pigmentation of the milk fat. This phase of the question will be given more consideration in a later paper.

*Experiment 7.* This was a second carrot feeding experiment, using another cow, *i.e.*, Holstein Cow No. 221. As in the foregoing experiment the ration was first changed to a non-pigmented one and when the color of the fat had dropped to about 8 units of yellow the cow was given all the carrots she would clean up. The experiment was not as successful as anticipated because the animal would not eat more than 10 pounds of the carrots per day except at the very last. As our supply of carrots was limited, the experiment was discontinued. It is interesting to note, however,

TABLE 9.

*The effect of a non-pigmented ration and of a ration containing carrots upon the color of milk fat. (Holstein Cow No. 221.)*

DATE OF SAMPLE	HAY*	CARROTS	COTTON- SEED MEAL	CORN	COLOR OF BUTTER FAT	
					Yellow	Red
	lbs.	lbs.	lbs.	lbs.		
1912	Normal herd ration containing green alfalfa hay					
November 29.....					26.0	1.5
December 9.....	16		4	4	8.0	1.4
December 17.....	16	10	4	4	6.0	1.3
December a.m. 25.....	16	20	4	4	14.0	1.5
December p.m. 25.. ...	16		4	4	20.0	1.5
January 3.....	16	0	4	4	7.5	1.5

\* The hay was a mixture of equal parts of light colored timothy and bleached alfalfa.

that only 10 pounds of carrots per day were sufficient to bring the color of the fat almost back to the starting point. The data are given in Table 9.

*Experiment 8.* This was a feeding experiment the results of which corroborated those of Experiment 1, showing that the color of the milk fat of Jersey cows is as much dependent upon the food as that of other breeds. In addition the experiment offers excellent proof that when the character of a non-pigmented ration is such that only the normal storage of pigment in the body, *i.e.*, the blood serum, is drawn upon, the drop in color is much more rapid than when the ration does not supply the nutrients required

for body maintenance and milk production as was the case in Experiment 1. It will be remembered that thirty days were required for a drop in color of 43 to 8.5 units of yellow, in Experiment 1. It will be noticed that even a greater drop took place in the present experiment in only twelve days. The data are given in Table 10.

TABLE 10.

*The effect of a non-pigmented ration on the color of milk fat. (Jersey Cow No. 59.)*

DATE OF FEEDING	GRAIN MIXTURE*	CORN SILAGE	HAY	DATE OF SAMPLE	COLOR OF BUTTER FAT		
					Yellow	Red	Light
1911	lbs.	lbs.	lbs.				
March 11 to April 2.....	11.6	0	14†	April 2	46	1.8	0.5
April 3 to April 14....	11.0	10	9‡	April 4	6	1.5	0.2
April 15 to April 23....	11.0	10	8	April 23	4	1.5	0.2
April 24 to May 8.....	11.0	10	4	May 9	3	1.5	0.2
May 10 to May 20.....	11.0	10	4	May 20	3	1.5	0.2
May 21 to June 14....	11.5	corn stover 5	8§	June 14	47	1.8	0.5
June 15 to June 29.....	12.0	corn stover 5	8	June 29	26	2.0	0.2

\* The grain mixture was 5 pounds of corn and 6 pounds of cottonseed meal.

† Green alfalfa hay from March 11 to April 2.

‡ Bleached timothy hay from April 3 to May 20.

§ Green alfalfa hay beginning May 21.

*The relation of the breed of the cow to the color of the milk fat.*

It is generally accepted as a fact that the breed of the cow has a pronounced relation to the color of the milk fat, and that the Jersey and Guernsey breeds rank first in this respect. This has been attributed to some inherent physiological quality which these breeds possess over other breeds.

The data which have already been presented show conclusively, however, that Jersey cows (and the same is unquestionably true of

Guernsey cows also) are subject to the same variations in the color of the milk fat, with a variation in the food, as other breeds. In addition we have made a careful study<sup>27</sup> of the color of the milk fat of the different breeds under certain comparative conditions with the result that even under conditions favorable for the production of the highest colored milk fat, the breed difference was found to be only relative. In addition we have found that under feeding conditions productive of only moderate pigmentation of the milk fat the breed differences disappeared almost entirely. This is also true of the colostrum pigmentation as was shown in Table 3 above, at which time there is no breed characteristic evident.

There is, however, one breed difference which has led probably more than anything else to the belief that Jersey (and Guernsey) cows are able to produce yellow butter fat at any time regardless of feed. This difference lies in the fact that there is usually a much greater storage of pigment in the body fat of Jersey and Guernsey cows than in the body fat of the other breeds. The significance of this difference was shown clearly in Experiment 1 above, where it required nearly thirty days to reduce the color of the butter fat of a Jersey cow from only a moderately yellow fat to a light colored fat on a non-pigmented ration.

Without going further into detail it may be said that our experiments have led us to conclude that the so-called breed characteristic is given more emphasis than is warranted by an actual study of the facts.

#### SUMMARY AND CONCLUSIONS.

1. The fat of cows' milk owes its natural yellow color to the pigments carotin and xanthophylls, principally carotin, the well known, wide spread, yellow vegetable pigments found accompanying chlorophyll in all green plants.

2. The carotin and xanthophylls of milk fat are not synthesized in the cow's body, but are merely taken up from the food and subsequently secreted in the milk fat.

3. When food practically free from carotin and xanthophylls such as the cow usually receives during the winter months, is given to a milk-giving cow, the immediate supply of these pigments in

<sup>27</sup> A detailed account of this study may be found in Research Bulletin No. 10, Missouri Agricultural Experiment Station.

the organism is greatly depleted and may be entirely used up, on account of the constant drain upon the supply by the milk glands. The butter fat accordingly approaches a colorless condition in proportion to the supply of carotin and xanthophylls in the system, the length of time these pigments are kept out of the food, and also very probably in proportion to the amount of milk fat being produced.

4. If food rich in carotin and xanthophylls is given to a milk-giving cow whose milk fat has become practically colorless by reason of the above conditions, the organism will at once recover its lost pigments and the milk fat will increase in color in proportion to the amount of carotin and xanthophylls, especially carotin, in the food. Fresh green grass probably being the richest in carotin of all natural dairy cattle feeds, accordingly produces the highest colored butter.

5. There is some difference among the different breeds of dairy cows in respect to the maximum color of the milk fat under equally favorable conditions for the production of a high color. Each breed of cows, however, will undergo the same variation in color of the milk fat which follows a withdrawal or addition of carotin and xanthophylls, especially carotin, to the food. Under some conditions, also, the apparent breed characteristic largely disappears. The popular opinion in regard to the breed characteristic has been overemphasized, and statements in regard to it should in the future be qualified with a statement of the conditions of the feed, etc.

6. Under normal conditions cows of all breeds produce very high colored milk fat for a short time after parturition. The pigments of the fat at this time are identical with the normal pigments of the fat. Their increase at this time is probably due to the physiological conditions surrounding the secretion of the milk of the freshening animal.

# CAROTIN—THE PRINCIPAL NATURAL YELLOW PIGMENT OF MILK FAT: ITS RELATIONS TO PLANT CAROTIN AND THE CAROTIN OF THE BODY FAT, CORPUS LUTEUM AND BLOOD SERUM.<sup>1</sup>

## II. THE PIGMENTS OF THE BODY FAT CORPUS LUTEUM AND SKIN SECRETIONS OF THE COW.

By LEROY S. PALMER AND C. H. ECKLES.

(From the Dairy Chemistry Laboratory, University of Missouri, in coöperation with the U. S. Department of Agriculture.)

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In the previous study<sup>2</sup> we have shown that the commonly observed yellow lipochrome of butter fat from cows' milk is composed principally of a pigment identical with carotin, the unsaturated hydrocarbon pigment widely distributed in plants. The carotin of the butter fat, like the carotin of plants, has associated with it one or more xanthophyll pigments, but in much smaller proportion than is usually found in green plants.

We have furthermore shown that these pigments are present in the milk fat as a result of feeding a ration containing an abundant amount of these pigments. The presence of carotin and xanthophyll in milk fat therefore is not due to any synthetic powers which the cow possesses, but merely to the fact that the organism absorbs the pigments along with the digestion products of the food and subsequently secretes them dissolved in the milk fat. We were accordingly able to vary the amount of pigment in the milk fat by choosing the proper feeds, *i.e.*, either deficient in carotin and xanthophylls or very rich in these pigments. The above relations between the carotin and xanthophylls of milk fat and the carotin and xanthophylls of the feed were found to hold good for all breeds of dairy cows.

The above results at once opened the question of a similar relation of the food pigments to the yellow pigment which is so

<sup>1</sup> Published by permission of the Secretary of Agriculture.

<sup>2</sup> This *Journal*, xvii, p. 191, 1914.

often observed in the body fat of cows, especially those of certain breeds, such as the Jersey and Guernsey. In addition the questions were raised, whether the carotin of the corpus luteum has associated with it the xanthophyll constituents found in milk fat; and whether the yellow pigment of the skin secretions of some breeds of cows is the same that characterizes the butter fat.

The investigation here reported was undertaken for the purpose of studying these questions.

#### EXPERIMENTAL.

The methods of studying and identifying the pigments were the macroscopic ones used in the preceding study of the lipochrome of milk fat, and may be found in detail in the paper dealing with that pigment. These methods were: 1. A careful differentiation of the pigment into its carotin and xanthophyll constituents by using their property of relative greater solubility in low-boiling-point petroleum ether and in 80 per cent alcohol respectively. 2. A further demonstration of the character of each constituent by studying its adsorption properties toward calcium carbonate, when in anhydrous carbon bisulphide. 3. A careful comparison of the spectroscopic absorption bands of each constituent obtained in (2) and (3) with the bands of carotin and xanthophylls of plants obtained in a similar manner.

#### *The pigments of body fat.*

The pigment of the body fat of cows has never been subjected to critical examination. Newbigin<sup>3</sup> reports the only attempt to identify it. He compared the pigment with a yellow non-lipochrome pigment which he isolated from the salmon, and also with the yellow pigment of maize. Newbigin concluded from his study that the body fat pigment, like the yellow salmon pigment was not a lipochrome. The maize pigment was similar in all respects except that it gave the lipochrome color reactions. On account of this result, we paid particular attention to the lipochrome properties of the body fat pigment.

The same method was used for isolating the body fat pigment as was used for the milk fat pigment. It consisted in careful

<sup>3</sup> Cf. D. Noël Paton, *Report of Investigations on the Life History of the Salmon*, 1898, Article XV.

saponification of the fat with alcoholic potash (2 cc. of 20 per cent solution per gram of fat) and subsequent extraction of the unsaponifiable matter from the diluted soap (three volumes of water to one of soap) with ether. The ether extract was sometimes purified by re-saponification and was sometimes freed from cholesterol with digitonin. Twenty-five to thirty grams of fat were used for isolating the pigment for study.

The results of the study of the pigment from several samples of high-colored body fat were uniform in showing that the principal pigment is, as in the case of milk fat, carotin. It is readily extracted from the 80-90 per cent alcohol by petroleum ether (b.p. 30°-50°C.) and cannot be extracted from the latter solvent by 80-90 per cent alcohol. The pigment gives a blood-red carbon bisulphide solution and in this solvent is unadsorbed by calcium carbonate. In carbon bisulphide as well as in alcohol, the pigment shows the characteristic absorption bands of carotin. The above properties were true only of the freshly prepared pigment, and in this state only showed the characteristic lipochrome color reactions with concentrated  $\text{H}_2\text{SO}_4$  and  $\text{HNO}_3$ .

Like the carotin of milk fat, the body fat carotin has associated with it one or more constituents which show the properties of the xanthophylls. They are readily extracted from low-boiling-point petroleum ether by 80 per cent alcohol and cannot be re-extracted from the latter solvent by the petroleum ether. In carbon bisulphide they are partially adsorbed by calcium carbonate and in the same solvent as well as in alcohol show the absorption bands of the xanthophylls, which are noticeably shifted toward the more refrangible part of the spectrum from the carotin bands. The measurements of the bands of both carotin and xanthophylls from body fat, made according to the arbitrary standard scale explained in the previous paper dealing with the milk fat pigment, are given in the following Table 1.

TABLE 1.

*Absorption bands of carotin and xanthophylls of body fat.*

BAND NO.	CAROTIN	BAND NO.	XANTHOPHYLLS
I.	224-242	I.	235-252
II.	262-288	II.	278-302
III.	302-322	III.	315-335

given in Table 2, and three Holstein cows. The data are given in Table 3.

TABLE 3.

*The relation of breed to the color of body fat.*

PART OF BODY	COW NO. 2 (Jersey)		COW NO. 207 (Holstein)		COW NO. 226 (Holstein)		COW NO. 221 (Holstein)	
	Yellow	Red	Yellow	Red	Yellow	Red	Yellow	Red
Rib plate.....	54	1.5	47	1.7			15	1.2
Crops.....	63	1.8	14	1.2			21	1.2
Thoracic cavity.....	17	1.0	36	1.5	9	1.0	6	1.2
Caul.....	50	1.7	54	1.7	18	1.1	12	1.2
Pelvic cavity.....	47	1.5	61	1.8			10	1.2
Over last rib.....	63	1.8	17	1.3			23	1.2
Ovaries, uterus.....			62	1.8				
Chuck.....	54	1.7	14	1.0			22	1.3
Kidney.....	47	1.5	64	1.8			20	1.2
On fourth stomach.....					24	1.0	11	1.2

The significant points brought out by the data in the above table are as follows: First, that it is possible to find pure bred Holstein cows with body fat of as high a color as is apparently normal to Jersey cows. Second, that the body fat of Holstein cows is often characterized by a much lower color than is apparently normal to Jersey cows. An interesting feature of this difference is that Holstein cow 207 was known to give high colored milk fat, while Holstein cow 221 never gave milk fat of a color which exceeded the maximum color found in her body fat even under the most favorable conditions for the production of a high color. Both of these cows were dry and barren at the time of slaughtering and had been fattened largely on pasture grass and grain, a ration rich in carotin. This result points to an individual variation, as well as a breed variation, in regard to the color of the body fat.

The relation between the maximum color of the body fat and milk fat which an individual cow will produce under a given feeding condition is an important one. It will be shown in a later paper that this maximum color, especially of the milk fat, is independent of the amount of carotin in the blood. This is also true in regard to the color of the body fat. One would ex-

pect this by analogy and it was found to be true from an experimental standpoint. Without entering into details here in regard to the method of analysis, which will be given in a later paper, it may be stated that the blood of Cow 221 was found to be as rich in carotin as is normal to a Jersey under the same conditions. These results point to the fact that whatever differences occur among individuals or breeds in regard to maximum coloration of milk fat or body fat, arise at the point of formation of the fat, i.e., at the milk glands and body cells.

*The pigments of the corpus luteum.*

Escher<sup>5</sup> has recently shown by chemical analysis that the pigment which can be crystallized from the high-colored corpus luteum of the cow is identical with the carotin of plants. In view of the fact that our investigations have shown that the lipochromes of the milk fat and body fat of the same animal are the same pigment and especially that they have associated with them one or more minor xanthophyll constituents, it became important to study the corpus luteum pigment in this connection also.

Only a few corpora lutea were available for this study, they being obtained at various times at the slaughtering of experimental cows at the experiment station. In some cases the cows had well developed corpora lutea and at other times only the remains of former corpora lutea were obtained. In each case, however, the pigmented tissue was carefully cut away and macerated with sand and the pigment extracted with ether. In some cases the extract was saponified before examining the pigment.

The result of several such studies completely confirmed the carotin properties of the principal pigment. In addition in two cases it was found that after saponification a minor portion could be extracted by 80 per cent alcohol from a petroleum ether solution of the entire pigment, which could not be reextracted by fresh petroleum ether. In another case where a complete petroleum ether extract of two ovaries, one of which had a very large corpus luteum, was studied, no xanthophyll pigments were obtained after saponification. We were unfortunate in not having sufficient corpora lutea to carry this study to completion.

<sup>5</sup> *Zeitschr. f. physiol. Chem.*, lxxxiii, p. 198, 1913.

Undoubtedly the normal corpus-luteum carotin sometimes has associated with it some xanthophyll, which is probably present in the fat which may be extracted along with the corpus-luteum carotin.

*The pigments of the waxy secretions in the ears and on the skin of Jersey cows.*

The skin secretion of Jersey (and Guernsey) cows is often characterized by a more or less yellow color. This is considered by the breeders of these cattle to indicate their ability to secrete yellow milk fat. It was thought that a brief investigation of this pigment might be of interest and possibly of some scientific value.

The yellow skin pigment is sometimes very abundant in the ears of these animals. A few grams of the yellow wax was scraped from the ears of several pure bred Jersey cows. The wax was macerated with ether, which readily dissolved the pigment. After saponification and extraction with ether, the pigment was submitted to a macroscopic examination with respect to its carotin and xanthophyll properties.

The result was that the pigment was readily divided into a major constituent which showed all the properties of carotin, and a very minor constituent which was a xanthophyll. It is thus seen that the pigments which characterize the skin secretions of the Jersey breed are the same pigments that are found in the milk fat and body fat.

*The body fat and blood serum pigments of the new-born calf.*

Carotin and xanthophylls having been found to be normal constituents of the body fat of cows which had received an abundance of these pigments in the food, an interesting question was raised whether these pigments are present in the body of the new-born calf. Should these pigments be absent from the new-born calf, additional proof would be obtained that their presence in the mature animal is the result of feeding. Should they be present in the new-born calf, it would merely indicate that they were able to traverse the placental barrier from the mother whose body is normally rich in carotin and xanthophylls. In this connection the question would be especially interesting in view of

the fact that Mendel and Danicls<sup>6</sup> have found that fat-soluble dyes, such as Sudan III, do not traverse the placental barrier of small animals such as cats and rats, whose milk fat and body fat, however, is readily tinted as the result of feeding the dyes.

In order to study this question, a new-born Jersey calf was not allowed to suckle its mother but was slaughtered a few hours after birth. The blood was caught in a cylinder and allowed to clot. The serum was carefully investigated for the presence of carotin. No pigment was found which could be precipitated from the serum with the proteins and extracted therefrom with alcohol. The filtrate also yielded no lipochrome-like pigments.

A very small amount of fat could be obtained from the body of the calf, in all weighing about 40 grams. The rendered fat had a very faint yellow color. On saponification, it yielded a small amount of unsaponifiable pigment which could be differentiated into two constituents which showed properties identical with carotin and xanthophyll respectively.

It appears that carotin and xanthophylls do traverse the placental barrier of the cow to a very slight extent. It is possible that their presence in the body fat was due to a direct transfer of pigmented fat from the mother to the foetus. On this ground the absence of the pigments in the blood could be explained.

### *Discussion of results.*

Viewing the results of the above studies from a physiological standpoint, it is readily seen that the establishment of the source of the yellow pigments of the body of the cow, and the ease with which they are therefore increased and decreased,<sup>7</sup> throws great doubt upon any physiological significance which they have been supposed to exert in the animal body. In the case of the corpus luteum for instance, the accumulation of carotin during the formation of this body is merely a phenomenon incidental to the rupture of the Graafian follicle and the subsequent formation of the cellular tissue around the central blood clot, and to the fact that the blood serum is normally very rich in carotin as will be shown in a subsequent paper.

<sup>6</sup> This *Journal*, xiii, p. 72, 1912.

<sup>7</sup> This is especially true of the milk fat and, as will be shown in a subsequent paper, the blood serum.

The popular opinion that the body fat of Jersey cows is normally characterized by a higher yellow color than Holstein cows has been at least partially confirmed by experimental study, although it was found that Holstein cows may also possess high-colored body fat. At least there seems to be more breed characteristic in this respect than in the case of the pigmentation of the milk fat. There is no foundation, however, for the belief that beef has a lower value because its fat has a high color. If this pigment is the same as is demanded by the consumer for butter, why should not beef with high-colored fat also be more desirable? It is recognized, of course, that some of the unfavorable attitude toward beef with highly colored fat arises partially from the fact that it indicates that the beef probably came from a dairy cow. The two ideas are nevertheless very closely associated.

The normally high color of the body fat of Jersey cows, and also of Guernsey cows explains why cows of these breeds often appear to be producing well colored butter on a ration deficient in carotin and xanthophylls. When cows whose body fat has a high yellow color are put upon a ration deficient in carotin and xanthophylls and also, as is usually the case with such rations, deficient in food value, the body fat is called upon to furnish energy for the animal and also in many cases to supplement the food digestion products in the production of milk fat. It is readily seen that in such cases an important source is opened up for pigments for the milk fat. The importance of this source would depend upon the amount of highly colored body fat available for the needs of the body, and upon the rapidity with which it would be used up. If our experimental data are correct showing that the inside fats, such as the caul fat and rib plate fat, are first drawn upon in starvation of this class of animals, then the amount of available highly colored fat would be rather large. Dairy cows usually have a fairly abundant amount of these fats, especially the caul fat. It is thus readily seen that a continuous drawing upon these inside fats for a long period of time would result in a very slow and gradual reduction of the color of the milk fat, especially since, as will be shown in a subsequent paper, the blood serum furnishes the principal, normal source of carotin for the milk fat. The deduction that the animal was actually producing colored milk fat on a carotin-xanthophyll-free ration would therefore be quite natural but nevertheless entirely false.

In a similar manner, it is readily seen why the breeders of Jersey and Guernsey cattle have been led to believe that the yellow skin secretions of these breeds are indicative of their ability to produce yellow milk fat. It is interesting to find that the yellow pigments of these secretions are carotin and xanthophylls. It should be clearly borne in mind, however, that the only indication that a cow will secrete yellow milk fat is that the food contains an abundance of carotin and xanthophylls.

#### CONCLUSIONS.

1. The yellow lipochrome of the body fat, corpus luteum, and skin secretions of the cow, like the lipochrome of butter fat, is composed principally of carotin, which sometimes has associated with it one or more minor xanthophyll constituents.

2. The carotin and xanthophyll pigments of the body fat are derived from the food of the cow. The body fat of Jersey cows formed on a ration deficient in carotin and xanthophylls is devoid of color.

3. The body fat of Jersey and Guernsey cows is usually characterized by a higher yellow color than the body fat of other breeds. This is of great importance in explaining why Jersey and Guernsey cows sometimes show a much slower elimination of pigment from the milk fat on a non-pigmented ration, as during the winter months. In these cases the body fat furnishes a supplementary source of carotin and xanthophylls for the milk fat.

4. The yellow body fat of Jersey and Guernsey cows should not be a point against the use of these animals for beef. The pigments here are the same as those for which the consumer will pay a higher price when present in butter.

5. The breeders of Jersey and Guernsey cattle are no doubt correct in their belief that the yellow skin and skin secretions of these animals are characteristic of these breeds. It is not correct, however, that this characteristic is indicative of the ability of the breeds to secrete yellow milk fat under all conditions. The only indication of this is whether the food contains an abundance of carotin and xanthophylls.

6. The blood serum of the new-born Jersey calf is free from carotin and xanthophylls. The small amount of fat on the body is tinted faintly with these pigments.



# CAROTIN—THE PRINCIPAL NATURAL YELLOW PIGMENT OF MILK FAT: ITS RELATIONS TO PLANT CAROTIN AND THE CAROTIN OF THE BODY FAT, CORPUS LUTEUM, AND BLOOD SERUM.<sup>1</sup>

## III. THE YELLOW LIPOCHROME OF BLOOD SERUM.

By LEROY S. PALMER AND C. H. ECKLES.

(From the Dairy Chemistry Laboratory, University of Missouri, in coöperation with the U. S. Department of Agriculture.)

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Practically no investigation of the lipochrome of the blood serum of the cow has been reported in the literature. Krukenberg<sup>2</sup> many years ago extracted the pigment from the blood serum of the ox, and identified it as a lipochrome. Thudichum<sup>3</sup> had a number of years earlier classified blood serum pigments of this character in general as luteins. Some work has been published in regard to luteins of the blood serum of fowls. Halliburton<sup>4</sup> believed that the lutein in the serum of the hen, dove, etc., was identical with the pigment of the fat of these animals. Schunck<sup>5</sup> later spectroscopically identified the lipochrome of the blood serum of the hen, with the L. xanthophyll which he isolated from yellow flowers and green plants.

Our own investigations<sup>6</sup> establishing the identity of the lipochromes of the milk fat, body fat, etc., of the cow with the carotin (chiefly) and xanthophylls of plants, led us to believe that the lipochrome of the blood serum of the cow is also composed of carotin and xanthophyll. It was recognized also that the establishment of such a fact would furnish the conclusive evidence of the

<sup>1</sup> Published by permission of the Secretary of Agriculture.

<sup>2</sup> *Sitz.-Ber. d. Jen. Gesellsch. f. Med.*, 1885.

<sup>3</sup> *Proc. Roy. Soc.*, xvii, p. 253, 1869.

<sup>4</sup> *Journ. of Physiol.*, vii, p. 324, 1886.

<sup>5</sup> *Proc. Roy. Soc.*, lxxii, p. 165, 1903.

<sup>6</sup> This *Journal*, xvii, pp. 191 and 211, 1914.

physiological relation between the food carotin (and xanthophylls) and the pigments of the milk fat, body fat, etc., especially if it could be shown that the lipochrome of the blood serum is subject to the same variations in quantity as were found to take place with the pigment of the milk fat and body fat.

The investigation here reported was undertaken for the purpose of studying the chemical and physiological relations of the lipochrome of the blood serum to the carotin and xanthophylls of the food and to these pigments as found in the milk fat. In addition, a careful study was made of the method by which the blood serum transports its lipochrome.

#### EXPERIMENTAL.

The methods of identifying the blood serum lipochrome were identical with those used for the identification of the milk fat and body fat pigments. A complete description of these methods may be found in connection with the report of the identification of the milk-fat pigments and need not be repeated here.

The method of isolation of the blood serum pigment was naturally different from that used in isolating the pigment from fat. As the result of considerable experimental work in this connection, the method which yielded the most satisfactory results was as follows: The clear serum, practically free from red corpuscles, which presses out when the freshly caught blood is allowed to clot without being disturbed, is completely desiccated with an excess of plaster of Paris. The powdery mass is then moistened to a thick paste with absolute or 98 per cent alcohol and thoroughly shaken with low-boiling-point petroleum ether (30°-50°C.) until no more color is extracted. The petroleum ether separates sharply from the pasty mass and may be directly poured away from it (through a filter if necessary). When the petroleum ether no longer extracts any color, any remaining fat-soluble pigment may be shaken out with ether, which readily mixes with the alcohol in the pasty mass. This alcohol-ether extract is then diluted with a large volume of water in a separatory funnel. Any pigment in the solution will rise to the top in the ether layer. After washing with water, the ether layer is added to the petroleum ether extract. Using the above simple procedure, a complete extraction of the lipochrome

pigments of the blood serum may be obtained. The purpose of the ether extraction, following the petroleum-ether extraction, will become clear in the experimental data which are to follow.

*Identification of the pigment.*

The method used for isolating the pigment from the serum indicates the character of the principal constituent of the pigment. Extracting the pigment from the alcohol-moistened, desiccated serum by means of petroleum ether, is equivalent to extracting the pigment from 80-90 per cent alcohol by petroleum ether, and indicates its carotinnature. The pigment which is extracted from the blood serum of the cow by this procedure is in fact identical in all its properties with the carotin of green plants and of the milk fat and body fat of the cow. In addition to showing the solubility properties of carotin, the pigment in carbon bisulphide solution (which has a blood-red color) and in alcohol shows the absorption bands of carotin. The pigment also, like carotin, shows no adsorption affinity toward calcium carbonate, but when its deep red, carbon bisulphide solution is filtered through a column of the adsorption material, it readily passes through as a rose colored zone.

The blood serum of the cow, like the milk fat and body fat, contains a small amount of xanthophyll pigments in addition to the main carotin pigment. In order to show the presence of the xanthophylls it is necessary to use a comparatively large amount of serum, *i.e.*, 200 to 300 cc., and make a complete extraction of the lipochrome pigments. It is in this connection that the second part, or ether extraction, of the method of isolation is important. When this is carefully done, and the pigments of the combined extracts are submitted to saponification and subsequently to macroscopic examination, it is found that a small portion of the pigments can usually be extracted by 80 per cent alcohol from their solution in petroleum ether. The pigment extracted by the 80 per cent alcohol cannot be reextracted by fresh petroleum ether. It, furthermore, gives an orange colored carbon bisulphide solution, shows the absorption bands of xanthophyll, and shows more or less adsorption affinity toward calcium carbonate.

The absorption bands of the carotin and xanthophylls of blood serum obtained from the serum of two different Jersey cows in two typical experiments are given in the following Table 1.

TABLE 1.

*Absorption bands of carotin and xanthophylls of blood serum.*

SOURCE OF SERUM	SOLVENT	ABSORPTION BANDS*	
		Carotin	Xanthophyll
Jersey cow, No. 8 .....	CS <sub>2</sub> {	I. 225-242 II. 263-286 III. 305-325	I. 232-254 II. 273-295 III. — — —
Jersey cow, No. 2 .....	CS <sub>2</sub> {	I. 223-242 II. 262-286 III. 300-320	I. 232-252 II. 270-292 III. 310- —

\* The bands were measured according to the fixed and arbitrary scale, which was described in our report of the study of the milk-fat pigment.

The difficulty of showing the presence of xanthophyll in the blood serum, in that quantitative extraction of all ether-soluble pigment and saponification of the extract is required, seems to indicate that a relation exists between the xanthophylls and the fat of the blood serum. Blood serum contains only a small proportion of fat, *i.e.*, less than 1 per cent, and the xanthophylls bear a similar relation to the total pigments of the serum.

*The physiological relation between the carotin of blood serum and the food of the cow.*

The relations between the amount of carotin in the blood serum, and the amount of carotin in the food, and in the milk fat being secreted, were studied by observing the amount of color that could be extracted from a definite volume of serum during various feeding experiments. These experiments were those described in an earlier<sup>7</sup> paper, which resulted in a variation in the color of the milk fat. In order that the results might be comparative, the procedure was as follows:

When the color reading of a 1-inch layer of melted butter fat (using the Lovibond tintometer) had reached any desired point as the result of any particular ration, a trocar was inserted in the jugular vein of the cow and 200 cc. of blood drawn into a cylinder. As soon as the blood had clotted and sufficient serum had pressed out, two 10-cc. portions were pipetted

<sup>7</sup> This *Journal*, xvii, p. 191, 1914.

off into a shallow dish and completely desiccated with an excess of plaster of Paris. The powdered mass was moistened with alcohol. The pigment was completely extracted at once, in one case with petroleum ether (b.p. 30-50°C.) and in the other case with ether. In each case the extract was carefully evaporated to a volume of 1 or 2 cc. (in the case of the ether extract it was first necessary to free it from alcohol with water), made up to a standard volume of 12.5 cc. with absolute alcohol, and the color of the solution determined in the Lovibond tintometer. The entire procedure was carried out as rapidly as possible. The results of the two determinations were averaged.

The first series of experiments were run during the feeding experiments of Ayrshire cow No. 301. These experiments were reported in detail in the previously mentioned paper.

The effect of the rations on the color of the milk fat and the color contained in 10 cc. of blood serum is shown in Table 2 below.

TABLE 2.

*The relation of the character of the ration to the color of the milk fat and blood serum.*

DATE OF SAMPLE	FEED OF COW	BUTTER FAT		SERUM	
		Yellow	Red	Yellow	Red
1913					
January 7....	Cottonseed meal and cottonseed hulls only.....	1.3	0.4	3.3	0.5
January 24...	Cottonseed hulls, timothy hay and white corn.....	1.2	0.4	2.6	1.1
February 7...	Timothy hay, cottonseed hulls, cottonseed meal and yellow corn.....	2.0	0.5	4.9	1.2
March 1.....	Ditto, plus 20 lbs. of carrots per day.....	24.0	1.3	54.0	1.8
March 6.....	Ditto.....	24.0	1.4	47.0	1.5
March 27.....	Ditto minus carrots.....	7.0	1.0	25.0	0.7

A similar special experiment was conducted with six other cows of different breeds. The data are given in Table 3. The first part of the data represents the effect on the color of the milk fat and blood serum of a long continued feeding of light colored timothy hay, corn stover and cottonseed meal, a ration very deficient in carotin and xanthophylls. The data in the second part of the experiment, taken thirty days later, represent the effect of chang-

ing the ration to one containing considerable very green colored alfalfa hay and some fresh pasture grass, a ration very rich in carotin and xanthophylls.

TABLE 3.  
*Relation of ration to color of milk fat and blood serum.*

COW NO.	BREED	DATE	FAT IN MILK	COLOR			
				BUTTER FAT		SERUM	
				Yellow	Red	Yellow	Red
			<i>grams</i>				
213	Holstein.....	3/11/13	122	8.5	1.4	6.0	0.7
213	Holstein.....	4/10/13	135	54.0	1.8	48.0	1.1
220	Holstein.....	3/11/13	167	3.0	0.7	7.0	0.8
220	Holstein.....	4/10/13	208	22.0	1.2	41.0	1.0
303	Ayrshire.....	3/11/13	213	2.5	0.6	11.0	0.9
303	Ayrshire.....	4/10/13	263	16.0	1.1	40.0	1.0
16	Jersey.....	3/11/13	304	11.0	1.7	10.0	0.9
16	Jersey.....	4/10/13	363	64.0	2.0	45.0	1.1
57	Jersey.....	3/11/13	240	5.2	1.2	13.0	1.1
57	Jersey.....	4/10/13	263	54.0	1.7	57.0	1.8
64	Jersey.....	3/11/13	281	4.7	1.5	7.5	0.7
64	Jersey.....	4/10/13	358	47.0	1.6	45.0	1.0

The data in Tables 2 and 3 show conclusively that the blood serum of the cow derives its carotin from the same source as the milk fat, i.e., the food. It also offers conclusive proof that it is the blood serum that transmits the carotin and xanthophylls from the food to the milk fat and body fat-producing cells.

Several other important points are brought out by the data in these tables. For instance, it is seen that an increase of pigment in the serum always means an increase of pigment in the milk fat, but a highly colored serum does not necessarily mean a highly colored milk fat. There seem to be other factors playing a part here that are not brought out by the data.

It is possible that the albumin content of the milk is an important factor in this connection. The data presented in Table 4 below, admit of such an interpretation. Additional evidence of this is the extremely high color of the fat of colostrum milk, which was pointed out in an earlier paper<sup>8</sup> and which is accompanied by an

<sup>8</sup> This *Journal*, xvii, p. 211, 1914.

abnormal amount of albumin in the milk. It should be mentioned, however, that the conditions surrounding the data in Table 4 were abnormal. The animal had been greatly underfed for a month previous to the time represented in this experiment. Her ration, however, had been moderately rich in carotin and xanthophylls. Her ration was changed on September 25 to a non-pigmented one, and was increased to an amount sufficient to bring the animal back to a normal plane of nutrition. This explains the increase in the ration as shown in the table. The point brought out is that although the conditions were favorable for a decline in the color of the milk fat, the reverse was the case; and the particularly important point is that the increase in color was coincident with a marked increase in the lactalbumin of the milk.

TABLE 4.

*A possible relation between the albumin of milk and color of fat.*  
(Ayrshire cow. No. 301.)

DATE	FEED		MILK FAT PER DAY	TOTAL PROTEIN PER DAY	CASEIN PER DAY	ALBUMIN PER DAY	COLOR OF FAT	
	Hay	Grain					Yellow	Red
1912	lbs.	lbs.	grams	grams	grams	grams		
9/23-24...	7.2*	4.9	254	209	138	37	15.0	1.8
9/25-26...	8.1†	4.9	249	203	134	36	15.0	1.8
9/27-28...	11.0	6.0	262	222	138	47	19.0	1.8
9/29-30...	14.0	7.0	272	230	135	57	21.0	1.7
10/1-2....	14.5	7.5	267	237	132	67	28.0	1.8
10/3-4....	9.8	8.0	276	220	139	46	20.0	1.7

\* Green alfalfa hay.

† Changed to bleached alfalfa hay.

An attempt to confirm this relation with twelve other animals, representing the Jersey, Holstein and Ayrshire breeds under normal conditions, resulted in failure. This result is not considered however as disproving the supposition of such a relation, for our knowledge of the subject is too limited at present to enable us to control all the factors that enter into the question.

*The transportation of the carotin and xanthophylls by the blood serum.*

It seemed to us a matter of considerable importance to ascertain how the blood carries the carotin and xanthophylls from the

to the fat-synthesizing cells of the mammary glands and body tissue.

It has already been shown that there is a strong possibility that the minor constituents of the pigments, *i.e.*, the xanthophylls, are carried in the serum dissolved in the fat. When the great volume of blood in circulation in the cow's body and the rapidity of circulation are considered, it seems very probable that even the small percentage of fat in the blood is sufficient to account for all the pigments; both carotin and xanthophylls, which are presented to the milk glands and body cells. On the other hand when the large quantity of carotin normally found in any given volume of serum is considered in relation to the amount of fat in that volume of serum, it must be concluded that the fat plays little, if any, part in the transportation of this pigment. The transportation of the major pigment, *i.e.*, the carotin, therefore offered the most interesting problem and we confined ourselves entirely to it.

If the carotin is not carried by the blood dissolved in fat, it seemed probable that it is carried in simple solution in the serum. Thudichum,<sup>9</sup> in fact stated that this is the case, and it seems very probable in view of the fact that Krukenberg<sup>10</sup> was able to dissolve the lipochrome out of ox serum by means of amyl alcohol. It is not possible, however, to show by experiment that this is the case. We treated freshly prepared residues of carotin from plants and from blood serum with fresh blood serum. There was no indication that the serum dissolved any of the carotin. The carotin itself was not in any way disturbed, and the serum did not show any increase in color that could be extracted by petroleum ether after desiccation with plaster of Paris and moistening with alcohol. In addition to this evidence, we have found that the carotin cannot be extracted from the serum by any of the solvents in which it is readily soluble, which do not first precipitate the proteins, which is the case with the alcohols. Little carotin<sup>11</sup> can be extracted from the blood serum on vigorous shaking with ether, petroleum ether or carbon bisulphide. Amyl alcohol will extract the carotin, because it first precipitates the proteins and furthermore because it does not mix with the serum. Ethyl and methyl alcohol do not

<sup>9</sup> *Loc. cit.*

<sup>10</sup> *Loc. cit.*

<sup>11</sup> Generally the extract is colorless.

extract the carotin from the serum. They will throw down the pigment with the proteins, and will then extract the pigment when warmed with the precipitated proteins.

On the other hand if the serum is first treated with alcohol, the carotin can be readily extracted by shaking with ether, petroleum ether or carbon bisulphide. Our method of extracting the carotin from the desiccated serum, as described above, was based upon this fact. If the desiccated serum is shaken with ether, petroleum ether, etc., alone, before treatment with alcohol, a mere trace of color can be extracted.

The above results seemed to point to a combination of the carotin with some substance from which it must be liberated before it can be extracted by its usual solvents. We have found this substance to be an albumin.

The evidence for this is based upon a large number of easily demonstrated facts, among which are the following. Blood serum rich in carotin can be diluted to any volume with water without liberating the carotin so that it can be extracted with ether, petroleum ether, etc. Carotin cannot be precipitated from the blood serum with the globulins by half saturating the serum with ammonium sulphate, or by saturating the serum with sodium chloride or magnesium sulphate. The carotin is thrown down only in those precipitates which contain the albumins, i.e., by saturation with ammonium sulphate, or by acidifying the magnesium-sulphate saturated serum with acetic acid to a concentration of 1 per cent, or by an excess of alcohol, as mentioned above, or by boiling the diluted serum. It is, furthermore, possible to roughly isolate the albumin which carries the carotin. To do this, the serum is first freed from globulins by adding an equal volume of saturated ammonium sulphate solution. A small amount of carotin which may be mechanically carried down by the globulins may be recovered by dissolving the precipitate in dilute ammonium sulphate solution and half saturating with ammonium sulphate. The filtrate is added to the first globulin-free filtrate. The combined filtrates are then carefully heated to a temperature of 79°C. and the coagulated albumins filtered off. This precipitate will contain a small amount of the carotin, but the greatest part is found in the filtrate. With a serum rich in carotin this filtrate will have a golden yellow color.

On complete saturation with ammonium sulphate the carotin-containing albumin is salted out. The precipitate will have a deep yellow color and will comprise but a small portion of the proteins which have already been precipitated. This pigmented protein is readily soluble in water. It does not give up its carotin to ether, petroleum ether, etc., until first treated with alcohol.

We have made a careful study of an aqueous solution of the carotin-carrying albumin prepared in the above manner from 700 cc. of blood serum, rich in carotin, obtained from a Jersey cow. The solution contained a small amount of ammonium sulphate. We found that the pigmented albumin could be precipitated from this solution by lead acetate, silver nitrate, and mercuric nitrate. These salts did not give up their carotin to petroleum ether until they were first treated with alcohol, and in the case of the lead and mercury salts only until they had soaked in alcohol for some time. The pigmented albumin was readily thrown down on boiling the solution or by saturating with ammonium sulphate or by adding an excess of alcohol. Petroleum ether alone would not extract the carotin from the last precipitate after it had been filtered off and allowed to thoroughly drain, but a mixture of absolute alcohol and petroleum ether readily extracted it.<sup>12</sup> This seems to indicate that the presence of alcohol is a necessary factor in our extraction method.

It was attempted to free the aqueous solution of the caroto-albumin from the ammonium sulphate by dialysis. An eight-day dialysis of the solution in a parchment bag against running water did not completely free the solution of the ammonium sulphate. On the other hand, a fine coagulation occurred in the solution. This was filtered off. It was insoluble in water. On treatment with ether it gave up an abundance of carotin, leaving the precipitate colorless. The ether solution thus obtained was tested for the presence of phosphorus-containing bodies. A pronounced test for phosphorus was obtained by the usual method, after fusing with NaOH and KNO<sub>3</sub>, etc.

It will be noticed that the rough isolation of the caroto- (or

<sup>12</sup> When in alcoholic solution, this extract gave a pronounced precipitate of digitonin-cholesteride on addition of hot 1 per cent digitonin solution in 90 per cent alcohol.

luteo-) albumin is based partly upon the fact that it is not coagulated to any extent in half saturated ammonium sulphate solution at a temperature of 79°C. The coagulation point of this solution was found to lie between this temperature and 86°C. The amount of albumin which is coagulated increases rapidly up to the higher temperature, but is coagulated completely only at 86°C. These facts point very clearly to the albumin character of the protein with which the carotin is combined. The upper limit of the coagulation temperature is somewhat higher than is usually given for serum albumin, but this is undoubtedly due to the fact that the protein is not a simple one, but a conjugated protein. A careful study was made of the coagulation temperature of this chromo-protein, isolated in the manner described above, in a normal and also in a half saturated (approximately 4 N) ammonium sulphate solution. The former solution first showed opalescence between 82° and 82.5°C. but would not coagulate even at 90°C. except when the solution was acid. The half saturated  $(\text{NH}_4)_2\text{SO}_4$  solution first showed opalescence between 79° and 80°C. and completely coagulated at 86°C.

We have no direct evidence of the character of the combination of albumin and carotin. It is evidently a firm one since it is so hard to break down, alcohol being the only reagent which we found would destroy the union so that the carotin could be extracted by its usual solvents, *i.e.*, ether, petroleum ether, carbon bisulphide, etc. The union is apparently broken down to some extent by dialysis, due no doubt to partial decomposition. It is specially interesting to note that cholesterol and a phosphorus-containing substance (probably lecithin) are mixed up in some way with the combination. This is probably the secret of the combination.

The finding of this highly unsaturated hydrocarbon pigment in combination with one of the albumins of the blood, calls to mind another pigmented protein of the blood, namely, the familiar haemoglobin of the red corpuscles. It is not probable however that this new chromo-protein is also important in the oxygen exchange of the body.

As already indicated, we propose the name caroto-albumin for the chromo-protein which transmits the carotin from the food to the milk glands and fat cells.

plays in the formation of the milk fat.<sup>13</sup> The same holds true for the body fat.

The readily demonstrated fact that the withdrawal of carotin from the food results in a marked decrease in the color of the milk fat being secreted or the body fat being formed, shows that the albumin which carries the carotin in the blood serum does play a definite part in the formation of both milk fat and body fat and no doubt also in the formation of the corpus luteum.

The whole phenomenon offers many interesting but difficult problems for future study. Many of these when solved, will undoubtedly throw much light upon the chemistry of the mechanism of milk secretion.

#### SUMMARY.

1. The well-known lipochrome of the blood serum of the cow is, like the lipochrome of the milk fat, body fat, etc., of the same animal, composed principally of carotin, the wide-spread hydrocarbon pigment of plants. Associated in small quantity with the carotin of the serum, probably dissolved in the fat of the blood, are one or more xanthophyll pigments which are always found in more or less variable quantity associated with the carotin of plants.

2. The carotin and xanthophylls of the blood serum are derived from the food and furnish the normal source for these pigments in the milk fat and body fat, etc. A variation in the quantity of these pigments in the food results in a corresponding variation in the amount found in the blood serum and milk fat. Body fat formed during this time will also be affected.

3. The carotin is carried by the blood serum in combination with an albumin. The combination is a very firm one. Lecithin and cholesterol are probably a part of the combination. We propose the name caroto-albumin for the new chromo-protein of the blood.

4. The caroto-albumin of the blood serum of the cow is probably of importance in the formation of milk fat, body fat and the corpus luteum of the cow. It is doubtful if this new pigmented protein is of importance in the oxygen respiration of the body.

5. The lactalbumin of cows' milk may, among other factors, be related to the color of the milk fat. There appears to be a special relation here in connection with the high color and high albumin content of colostrum milk.

<sup>13</sup> The presence of both cholesterol and lecithin in the caroto-albumin may explain the origin of these lipoids, as well as the carotin in butter fat.

# CAROTIN—THE PRINCIPAL NATURAL YELLOW PIGMENT OF MILK FAT: ITS RELATIONS TO PLANT CAROTIN AND THE CAROTIN OF THE BLOOD SERUM, BODY FAT AND CORPUS LUTEUM.<sup>1</sup>

## IV. THE FATE OF CAROTIN AND XANTHOPHYLLS DURING DIGESTION.

BY LEROY S. PALMER AND C. H. ECKLES.

*(From the Dairy Chemistry Laboratory, University of Missouri, in coöperation with the U. S. Department of Agriculture.)*

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Among the many interesting questions that have grown out of the establishment by us<sup>2</sup> of a chemical and also a physiological relation between the carotin and xanthophylls of plants and the yellow lipochrome of the milk fat, body fat, blood serum and corpus luteum of the cow, one which seemed to offer considerable physiological interest was why the carotin of the food plays the more important part in the pigmentation of the above named substances. Xanthophylls are as widely distributed in green plants as carotin. What is the reason that they appear in such small quantity in the blood serum and fat formations of the cow, while carotin is found abundantly in these substances whenever the ration is rich in carotin and xanthophylls?

Several methods of study, which did not appear to offer many difficulties, seemed available, by means of which considerable light might be thrown on this question. The results, unfortunately, were not as satisfactory as was expected. Some interesting points were brought out, however, which are sufficiently related to warrant the advancement of a fairly acceptable theory in regard to the question. The results are here presented since we did not have opportunity to study the question further.

<sup>1</sup> Published by permission of the Secretary of Agriculture.

<sup>2</sup> This *Journal*, xvii, pp, 191, 211, 223, 1914.

## 238 Fate of Carotin and Xanthophylls in Digestion

*Methods of study.* The methods of study referred to above were: 1. A study of the action of the various digestive fluids, both natural and artificial, upon fresh crude residues of the amorphous carotin and xanthophylls from fresh green plants. 2. A study of the character of the unsaponifiable pigment extracts at various places along the digestive tract of the cow. 3. A study of the character of the unsaponifiable yellow pigments excreted in the feces under conditions where unassimilated or undestroyed carotin and xanthophylls of the food would be likely to appear unchanged in the feces.

### EXPERIMENTAL.

#### *The action of digestive juices.*

The following solutions were added to equal portions of carotin and xanthophylls in test tubes, and the tubes plugged with cotton and set aside at 40°C. Observations for signs of decomposition were made every day for five days.<sup>3</sup>

<sup>3</sup> The carotin and xanthophylls were isolated as follows: 200 grams of air-dried, powdered, green alfalfa leaves were shaken with 3 liters of 10 per cent alcoholic petroleum ether for two days, and then with 1 liter of CS<sub>2</sub> until the solvent had taken up as much pigment as possible. The carotin and xanthophylls were isolated from each extract and combined. Each solution was now concentrated to 50 cc. and divided into ten parts. These were put into test tubes and the solvent driven off at a low temperature. The residues were used for the study reported above.

The carotin and xanthophylls were isolated from the alcoholic petroleum ether extract as follows: The xanthophylls were removed from the extract by shaking with an equal volume of 80 per cent alcohol. The carotin in the petroleum ether was now freed from chlorophylls by shaking with an excess of CaCO<sub>3</sub>, the solution was now evaporated into alcohol and transferred to ether by diluting with much water after the addition of ether. This solution was freed from traces of chlorophyll which had escaped adsorption by the CaCO<sub>3</sub> by shaking with 30 per cent alcoholic potash. The ether was then freed from alkali with distilled water. This ether solution of carotin was combined with the similar solution obtained from the CS<sub>2</sub> extract as described below. The 80 per cent alcohol, containing the xanthophylls, was partially freed from chlorophyll by shaking with moist animal charcoal for one hour. The pigments were then transferred to ether, and the remainder of the chlorophyll removed by 30 per cent alcoholic potash as in the case of the carotin. The ether solution was then

- Tube 1. 5 cc. of 0.25 per cent HCl solution of pepsin.  
Tube 2. 5 cc. of 0.25 per cent HCl solution of filtered gastric juice from the fourth stomach of a Jersey cow.  
Tube 3. 5 cc. of 0.25 per cent  $\text{Na}_2\text{CO}_3$  solution of trypsin.  
Tube 4. 5 cc. of 0.25 per cent  $\text{Na}_2\text{CO}_3$  solution of extract from pancreas of a Jersey cow.  
Tube 5. 5 cc. of 0.25 per cent  $\text{Na}_2\text{CO}_3$  solution of trypsin plus 5 cc. of fresh bile from a Jersey cow.  
Tube 6. 5 cc. of 0.25 per cent  $\text{Na}_2\text{CO}_3$  solution\* of pancreatic extract plus 5 cc. of fresh bile.  
Tube 7. 5 cc. of neutral solution of pancreatin.  
Tube 8. 5 cc. of neutral pancreatic extract.  
Tube 9. 5 cc. of neutral pancreatin solution plus 5 cc. of fresh bile.  
Tube 10. 5 cc. of neutral pancreatic extract plus 5 cc. of bile.  
The pepsin, trypsin and pancreatin were Merek's U. S. P. preparations.

A set of ten tubes were also prepared containing equal portions of the xanthophylls of yellow corn.<sup>4</sup>

The following results were obtained. *Carotin*: Bleaching occurred only in the tubes containing neutral and alkaline pancreatic extracts. In the same tubes plus bile there was no decoloration. The bile had no solvent action on the carotin, which was in marked contrast to the xanthophylls, as noted below. *Xanthophylls*: The pigments in tubes 1, 3, and 4 were largely decolorized at the end of the second day, while those in tubes 2, 7 and 8 retained their color after the fifth day. No observations could be made on the tubes containing bile until the fifth day on account of the fact that the bile had completely dissolved the pigments as soon as it was added. The pigments were examined by desiccating the contents of the tubes with plaster of Paris and extracting with ether. Marked bleaching had occurred in all the bile tubes. *Corn xan-*

washed free from alkali and added to the xanthophylls obtained from the  $\text{CS}_2$  extract as described below.

The carotin and xanthophylls were isolated from the  $\text{CS}_2$  extract as follows: The extract was concentrated into 95 per cent alcohol and after filtering was saponified with KOH. The pigments were extracted from the soap with ether. The ether was washed free from alkali and evaporated into alcohol. The carotin and xanthophylls were separated by differentiation between petroleum ether (b.p. 30-50°C.) and the alcohol.

<sup>4</sup>This was the unsaponifiable pigment of the corn which was more soluble in 80 per cent alcohol than in petroleum ether. A study of this pigment was made because we have found it to have no value in the pigmentation of milk fat.

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*thlophylls*: There was marked destructive action of these pigments in all the tubes except those containing bile. The corn xanthophylls, like the xanthophylls from the alfalfa, were readily soluble in bile.

The most significant feature of the above results is the marked difference in the solubility of carotin and xanthophylls in bile, the surprising result being the very slight solubility of the carotin. This was confirmed quantitatively using the carotin from another source and the bile from several different cows. The results are given in Table 1. The carotin used was a freshly prepared ether solution of carotin from carrots. Equal volumes of this solution were evaporated at a low temperature and the residues treated with 10 cc. of bile from each of four cows. After standing for several days with frequent shaking the bile was filtered and 5 cc. of the filtrate desiccated with plaster of Paris. This was extracted with ether until colorless. The extract in each case was concentrated to a low volume, made up to 12.5 cc. with absolute alcohol, and the color of the solution measured in the Lovibond tintometer.

TABLE 1.  
*The solubility of carotin in bile.*

EXP. NO.	SOURCE OF BILE	CAROTIN USED		CAROTIN IN BILE		BLANK*	
		Yellow	Red	Yellow	Red	Yellow	Red
1	Jersey.....	57	2	3.0	0.6	1	0.2
2	Angus.....	57	2	9.0	0.8	1	0.2
3	Holstein...	57	2	10.0	0.9	1	0.2
4	Holstein...	57	2	10.5	1.0	1	0.2

\*The blank is the amount of color extracted from the 5 cc. of bile alone, after desiccation with plaster of Paris.

An interesting feature in the above table is the apparent greater solubility of carotin in the bile of Holstein cows than in the bile of Jersey cows. No significance can be attached to this, however, until it can be confirmed.

### *The character of the pigments along the digestive tract.*

The plan in this part of the study was to examine the pigments which could be extracted from the material in various places along the digestive tract of several cows. Material was obtained from

one Holstein and two Jersey cows at slaughtering, from each of the four stomachs just before the food entered the next part of the digestive tract, from three places in the small intestines, from the caecum, and from the large intestine. One or two hundred grams of material were either dried on the steam bath or desiccated with plaster of Paris, and the resulting mass in either case extracted with  $\text{CS}_2$ . The solubility, spectroscopic, and adsorption properties of the extracted pigments were carefully noted. The pigments were thus differentiated into carotin and xanthophyll constituents as well as classified as belonging to either of the two groups.

The results of the study were not satisfactory, in that there was no uniformity among the several cows in regard to the character of the pigments found at any particular place although all the animals were receiving a ration which should have furnished an excess of both carotin and xanthophylls. The reason for this is not obvious. It might be thought that the partial drying in some cases destroyed the pigments. No doubt this occurred to some extent, but it would not account for the lack of uniformity where this method of desiccation was not employed.

No further discussion will be given this study. Mention has been made of it merely because the method of study seems to be a valuable one, and the study will bear repetition.

#### *The excreted pigments.*

For this study the feces of a cow were examined whose carotin and xanthophylls were furnished only by the feeding of carrots. The balance of the ration was composed of grain and timothy hay almost free from carotin and xanthophylls.

The method of demonstrating the character of the pigments in the feces was to desiccate a quantity of fresh feces with plaster of Paris and extract the mass with pure carbon bisulphide. The extract was concentrated and studied spectroscopically, and also by means of a Tswett chromatograph. The relative solubility properties of the pigments thus found were studied and also the spectroscopic properties of the pigments thus separated.

In this way it was found that when the cow was receiving 50 pounds of carrots per day, both carotin and xanthophylls were abundantly present in the feces. This continued for six days after

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the carrots were withdrawn from the ration, although it was possible to detect but little xanthophyll during this time.

### *Discussion of results.*

Combining the results of the above experiments, the appearance of carotin in the system when fed in excess may be explained on the ground of its greater stability toward the digestive processes as shown by the digestion experiments and the abundant appearance of the pigment in the feces. The failure of the xanthophylls to appear to any extent in the system may be due similarly to the fact that they are apparently more easily destroyed<sup>5</sup> during digestion. Some of them which escape destruction are undoubtedly taken up by the bile and thus enter the system through the portal circulation. Some oxidation probably takes place in the liver. If fat is present to any extent some of the xanthophylls will undoubtedly be taken up and enter the circulation dissolved in fat. In this connection it is of interest to note that we have shown in an earlier paper<sup>6</sup> that there is evidence to show that what xanthophylls can be found in the blood are present dissolved in fat.

An additional possible explanation of this whole question should not be overlooked, namely, that the difference in the proportion of carotin and xanthophylls taken up by the cow's body may be due to the difference in chemical composition between carotin and xanthophylls. Carotin is an unsaturated hydrocarbon and is furthermore capable of combining with a protein of the blood as we have shown in an earlier paper.<sup>7</sup> The xanthophylls, on the other hand, are carbon, hydrogen and oxygen compounds, in fact are chemically carotin dioxide. Although still unsaturated bodies, their slight difference in composition from carotin, may prevent their combination with the serum albumin, thus making it impossible for them to appear to any extent in the blood and fatty formations

<sup>5</sup> Willstätter and Miég (*Ann. d. Chem.*, ccciv, p. 1, 1907) state that xanthophylls are very sensitive towards acids. This would lead one to expect that they would be largely destroyed by the gastric juice. Our results were contradictory in this respect. We found an artificial gastric juice to destroy the xanthophylls but the natural gastric juice from the fourth stomach of a cow apparently had no effect on them.

<sup>6</sup> This *Journal*, xvii, p. 211, 1914.

<sup>7</sup> *Ibid.*

of the cow's body. If fat played a greater part in the food of the cow, the xanthophylls would undoubtedly appear to greater extent in the body of this animal.

#### SUMMARY.

1. Carotin is assimilated from the food of the cow in preference to xanthophylls partly because of its greater stability toward the juices of the digestive tract. Xanthophylls are much more soluble in bile than carotin,<sup>8</sup> which probably accounts for their appearance in the fat of the blood.

2. It is probable that carotin forms by far the greater part of the lipochromes of the cow's body chiefly on account of its ability to form a compound with one of the proteins of the blood. The xanthophylls, being of a different composition, probably are not capable of forming such a compound.

<sup>8</sup>A confirmation of the very slight solubility of carotin in bile is seen in the recent finding of Fischer and Röse (*Zeitschr. f. physiol. Chem.*, lxxxviii, p. 331, 1913) that the gall stones of cows contain crystallizable carotin.



# CAROTIN—THE PRINCIPAL NATURAL YELLOW PIGMENT OF MILK FAT: ITS RELATIONS TO PLANT CAROTIN AND THE CAROTIN OF THE BLOOD SERUM, BODY FAT AND CORPUS LUTEUM.<sup>1</sup>

## V. THE PIGMENTS OF HUMAN MILK FAT.

By LEROY S. PALMER AND C. H. ECKLES.

(From the Dairy Chemistry Laboratory, University of Missouri, in coöperation with the U. S. Department of Agriculture.)

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Our previously published studies<sup>2</sup> showing the chemical and physiological relations of the pigments of the fat of cows' milk to the carotin and xanthophylls of plants naturally opens the question whether the pigments which characterize the fat of the milk of other animals are of a similar character. Opportunity was not afforded to study this question with any domestic animals other than the cow. We fortunately had the opportunity, however, of investigating the character of the pigments which sometimes give a high color to the fat of human milk.

The methods used for studying the character of the pigments were the macroscopic ones used in our preceding studies. The adsorption properties were not studied, however, the demonstration being confined to the observation of the absorption bands and the relative solubility properties.

The fat from two samples of human milk from different sources was used. Very little was known in regard to one of the samples, it having been sent to the laboratory for analysis by a well-known physician of the community. The other sample was taken by one of us from a woman who had just given birth to a child, and represented a portion of the milk of each day of the first few days of lactation. Some further observations in regard to this sample will be reported below.

<sup>1</sup> Published by permission of the Secretary of Agriculture.

<sup>2</sup> This *Journal*, xvii, pp. 191, 211, 223, 237, 1914.

## EXPERIMENTAL.

*Experiment 1.* This was the sample in regard to which very little was known, with the exception that it was a bona fide sample of human milk. The milk had a faint yellow tint. The volume of milk used was approximately 125 cc. The milk contained about 3.5 per cent fat and therefore yielded a little over 4 grams of fat. The fat was obtained from the milk by precipitating it along with the proteins. To do this the milk was acidified with acetic acid, a pinch of salt added, and the milk brought to a boil. The precipitated proteins, when filtered off, had a bright yellow color, due to occluded fat. The fat was dissolved out with hot 95 per cent alcohol.

After concentrating the alcoholic extract, the fat was saponified by adding a small piece of KOH and boiling for about one hour. The pigment was readily extracted from the soap by ether, after dilution with water. The golden-yellow ether solution was washed with water and evaporated to dryness. The residue dissolved at once in carbon bisulphide with a red-orange color and in this solution showed two beautiful absorption bands, and possibly a third. The  $\text{CS}_2$  was carefully evaporated. A part of the residue which remained was difficultly soluble in absolute alcohol, but readily dissolved when a little petroleum ether was added. When differentiated between petroleum ether and 80 per cent alcohol the combined pigment was readily divided into two apparently equal portions with perhaps slightly more color in the petroleum ether layer.

The pigment of the petroleum ether layer gave a red-orange, carbon-bisulphide solution showing two strong absorption bands and a third faint one, the measurements of which are given in Table 1 below.

The pigment of the alcoholic layer gave a yellow-orange, carbon-bisulphide solution showing two good absorption bands and end absorption, the measurements of which are given in Table 1.

*Experiment 2.* As stated above, this sample of human milk was taken by one of us, and represented the milk of the first few days of lactation, including the colostrum milk. The milk itself was characterized by a high yellow color and the fat which

rose to the top of the sample had a very deep yellow color. About 350 cc. of milk were obtained. The fat percentage being between 5 and 6, nearly 20 grams of fat were yielded for the study of the pigments.

The fat was obtained from this sample of milk in a manner very similar to that used in the preceding experiment. The proteins and fat were precipitated together by adding a little salt and also considerable ammonium sulphate, acidifying with acetic acid and bringing to a boil. The precipitate was filtered off on a Büchner funnel. The layer of protein and fat had a golden yellow color. The fat was extracted with hot alcohol and ether. The golden colored extract was evaporated to dryness and the fat dissolved

TABLE 1.

*Absorption bands of carotin and xanthophylls from human milk fat.*

EXP. NO.	MEASUREMENTS OF ABSORPTION BANDS	
	Carotin	Xanthophylls
1	I. 225-244	I. 234-253
	II. 262-280	II. 275-293
	III. 300-319	III. 320- —
2	I. 225-242	I. 232-252
	II. 265-282	II. 273-293
	III. — — —	III. 312-330

away with ether. Alcohol was added and also 5 grams of KOH and saponification of the fat allowed to proceed on the steam bath for one-half hour. The pigment was extracted from the diluted soap with ether. After thorough washing with distilled water, the ether was evaporated carefully to dryness. The residue had a deep red color. It dissolved at once in petroleum ether (b.p. 30°-50°C.)

The pigment in this solution was now differentiated between the petroleum ether and 80 per cent alcohol. In this way it was divided into two portions which were about equal as far as could be detected by the color of the two solutions, with perhaps slightly more color in the 80 per cent alcohol.

The pigment in the petroleum ether layer gave a blood-red

colored carbon-bisulphide solution which showed two absorption bands and considerable end absorption. The measurements of these bands are given in Table 1.

The pigment in the 80 per cent alcohol layer gave an orange colored carbon-bisulphide solution which showed three distinct absorption bands. The measurements of these bands<sup>3</sup> are given in Table 1.

### *Discussion of results.*

The results of the above experiments show very clearly that the fat of human milk may be tinted with the same pigments found in the fat of cows' milk. The relative proportion of carotin and xanthophylls in human milk fat is much more nearly equal than in the fat of cows' milk. This is not surprising when it is considered that there is strong evidence that the xanthophylls are conveyed through the body dissolved in fat, and when it is also considered that fat plays a much greater part in human food than in the food of the cow.

An especially interesting fact brought out by these brief studies is that colostrum milk fat of the human is characterized by a very high color just as is the case with the fat of the colostrum milk from cows. In the experiment here reported, one of us had occasion to observe that after about ten days the milk fat from the same woman was very much lighter in color than during the first few days of lactation. The milk was also observed at intervals for a period of several months. A considerable variation in the color of the fat was noticed. Although it was not possible to accurately trace the cause of this variation as we did in the case of cows in an earlier paper of this series, it was undoubtedly due to changes in the diet.

In conclusion it may be stated that all students of human anatomy are familiar with the fact that the fat on the human body is often characterized by a marked yellow color. In view of the fact that the pigments of the milk fat and body fat of the cow are identical it must therefore be concluded that the pigments of the milk fat and body fat of humans are also identical.

<sup>3</sup> The bands were measured according to the arbitrary scale used in the preceding studies. A more detailed description of this may be found in the first paper of this series.

## SUMMARY.

1. The fat of human milk may be tinted by carotin and xanthophylls, the pigments which characterize the fat of cows' milk. The relative proportion of carotin to xanthophyll in human milk fat is much more nearly equal than in the fat of cows' milk.

2. The colostrum fat of human milk is characterized by a very high color as is the case with the fat of the colostrum milk of cows.

3. The pigment of human body fat is no doubt identical with the pigment of human milk fat.



# LACTOCHROME—THE YELLOW PIGMENT OF MILK WHEY: ITS PROBABLE IDENTITY WITH UROCHROME, THE SPECIFIC YELLOW PIGMENT OF NORMAL URINE.<sup>1</sup>

PLATES I AND II.

By LEROY S. PALMER AND LESLIE H. COOLEDGE.

(From the Dairy Chemistry Laboratory, University of Missouri in coöperation with the U. S. Department of Agriculture.)

(Received for publication, January 23, 1914.)

The natural yellow color of cows' milk is caused by two entirely different kinds of pigments: The principal one of the two pigments is found in the milk fat. It has recently been found by one of us<sup>2</sup> to be a mixture of carotin and xanthophylls, principally carotin, which are transmitted to the milk fat from the green feeds received by the cow.

The secondary or minor yellow pigment of milk has not been identified. Its presence in the milk is largely masked by the white color of the caseinogen and is only seen in the whey which remains after the caseinogen has been coagulated. The pigment is then seen imparting the usual greenish yellow color to the whey. This is a familiar phenomenon to the cheese maker.

The investigation here reported was undertaken for the purpose of establishing the relation of the whey pigment to other well-known animal pigments of similar character and also for the purpose of studying its importance in the pigmentation of milk.

A number of years ago Blyth<sup>3</sup> isolated a pigment from the whey of cows' milk. He described the free pigment as, "A bright red-orange, resin-like mass, softening at 100°C., very soluble in hot alcohol but partially separating on cooling." A concentrated solution of the pigment showed no spectroscopic absorption bands but merely a simple absorption of the

<sup>1</sup> Published by permission of the Secretary of Agriculture.

<sup>2</sup> This *Journal*, xvii, p. 291, 1914.

<sup>3</sup> *Trans. Journ. London Chem. Soc.*, 1879, p. 530; *Foods, Their Composition and Analysis*, 1896, 4th Edition, p. 239.

blue and violet. Blyth analyzed the mercury salt and gave it the formula  $\text{HgOC}_6\text{H}_5\text{NO}_2$ . He called the pigment lactochrome. He erroneously believed that this pigment also caused the yellow color of butter. A number of text-books have adopted this belief and use the name lactochrome to designate the yellow pigment of butter.<sup>4</sup>

Desmoulière and Gautrelet<sup>5</sup> recently studied a pigment which they isolated from milk whey and concluded that it was identical with urobilin. According to these authors if the slightly cloudy, greenish yellow, milk whey is acidified with sulphuric acid and saturated with ammonium sulphate, the greenish-yellow color is entirely precipitated along with the proteins; and the precipitate will yield to 90 per cent alcohol a yellow pigment with a greenish fluorescence, which after acidifying will show the characteristic absorption band of urobilin.

These authors also erroneously associated the whey pigment with the pigment of the milk fat. The isolated pigment did not give the characteristic color reactions of a lipochrome with concentrated sulphuric and nitric acids. The investigators accordingly concluded that no lipochromes exist in milk.

#### EXPERIMENTAL.

A study of the investigations of Blyth and of Desmoulière and Gautrelet makes it evident that they were both dealing with a specific pigment of milk whey and very probably with the same pigment.

We have a number of times carried out the isolation of lactochrome according to the method given by Blyth. The pigment which we isolated conformed in every respect to the description given by Blyth. We did not make any chemical analyses of the substance.

We also attempted to repeat the work of Desmoulière and Gautrelet, but without success. Contrary to their observations we found that only a very small portion of the whey pigment can be precipitated by acidifying the whey with sulphuric acid and saturating with ammonium sulphate; we also found that the pigment which can be extracted from the precipitate with alcohol does not show the absorption band of urobilin, but is spectroscopically inert. In addition we found that the greenish yellow filtrate

<sup>4</sup> Oliver: *Milk, Cheese and Butter*, p. 44; Lewkowitsch: *Chemical Technology and Analysis of Oils, Fats and Waxes*, 1909 Edition, Vol. II, p. 667; Wing: *Milk and Its Products*, 1912 Edition.

<sup>5</sup> *Compt. rend. soc. biol.*, lv, p. 632, 1903; Cited by Kastle and Roberts: *Hygienic Laboratory Bulletin*, No. 56, p. 319.

which is obtained after saturating the whey with ammonium sulphate readily gives up its color on mixing the solution with the proper quantity of absolute or 98 per cent alcohol. An alcohol layer containing all the pigment is obtained in this way.<sup>6</sup> The impure alcoholic solution of the whey pigment shows no absorption bands and does not show the characteristic urobilin fluorescence on treatment with ammonia and zinc chloride. The pigment, however, like lactochrome is readily precipitated by mercuric nitrate and silver nitrate. That the pigment obtained in this manner is identical with the lactochrome of Blyth is shown by the fact that when an aqueous solution of the lactochrome isolated by Blyth's method is saturated with ammonium sulphate only a small portion is precipitated and the remainder in the filtrate can be extracted by alcohol in the manner described above. The extract obtained in this way shows none of the properties of urobilin.

From the results of our study up to this point we felt justified in concluding that the lactochrome first isolated by Blyth is the cause of the greenish-yellow color of milk whey. As noted above we were not able, however, to detect any resemblance between this pigment and urobilin. On the other hand, to any one familiar with the urinary pigments there is at once a striking similarity between the general properties of the lactochrome of the whey and the urochrome of urine. Both pigments are readily soluble in water and ordinary alcohol but less soluble in absolute alcohol: Both form metallic salts of silver, mercury and copper: Both pigments are spectroscopically inert: And they both may be isolated from their mother solutions in the same manner, *i.e.*, by saturating the milk whey or urine with ammonium sulphate and extracting with absolute alcohol. The extracts obtained in this way from the whey are identical in appearance with similar extracts from urine.

*The action of "active" acetaldehyde on lactochrome and urochrome.*

Although the general properties of the whey pigment very closely resemble those of urochrome we did not feel justified in

<sup>6</sup> Desmoulière and Gautrelet extracted the pigment from whole milk: this manner, but interpreted the result in favor of urobilin.

concluding that the pigments are identical without submitting the whey pigment to a more critical examination. A comparative study of the elementary composition of the two pigments would have been ideal. It was not attempted, however, partly on account of the great difficulty attending the isolation of the pigments in a degree of purity sufficient for elementary analysis, but chiefly on account of the fact that none of the investigations of the elementary analysis of urochrome which have already been made have been found to agree. A solution of the question of the relation of lactochrome to urochrome was therefore sought in applying several additional characteristic urochrome tests to the whey pigment.

Garrod<sup>7</sup> found that when either pure or crude alcoholic solutions of urochrome were allowed to stand in contact with a small quantity of "active" acetaldehyde in the warm sunlight or on the steam bath, the appearance and properties of the solutions rapidly became identical with solutions of urobilin. The solutions which had previously been spectroscopically inert now showed the characteristic absorption band of urobilin and in addition showed the characteristic green fluorescence and broad band of "urobilin zine" after treatment with ammonia and zinc chloride. These changes were accompanied by a change of color to deep orange to red; and if allowed to continue the reaction was characterized by the appearance of a second absorption band in the blue.

Dombrowski<sup>8</sup> failed to confirm this action of "active" aldehyde on the urochrome prepared by his own method. Hohlweg<sup>9</sup> was more successful in applying the aldehyde test. He did not, however, observe any absorption bands, but his urochrome solution, "After a short treatment with pure acetaldehyde and warmth and subsequent addition of ammoniacal zinc chloride solution, showed an extraordinary strong green fluorescence, not at once, but after standing forty-eight hours in the air."

A close study of Garrod's paper setting forth the action of "active" aldehyde led us to conclude that his results cannot well be doubted; and that the failure of Dombrowski and Hohlweg to confirm Garrod's work was probably due to the fact that they did not have a truly "active" aldehyde solution.

<sup>7</sup> *Journ. of Physiol.*, xxi, p. 190, 1897; xxix, p. 335, 1903.

<sup>8</sup> *Zeitschr. f. physiol. Chem.*, liv, p. 183, 1908.

<sup>9</sup> *Biochem. Zeitschr.*, xiii, p. 199, 1908.

Two solutions of "active" acetaldehyde were prepared by us. One of these was a crude solution which we distilled in the usual way from the oxidation of alcohol by potassium dichromate and  $\text{H}_2\text{SO}_4$ . The other solution was a preparation of Merck's c. p. acetaldehyde sufficiently diluted with absolute alcohol to prevent its rapid volatilization. These solutions were "activated" by allowing them to stand in the strong hot sunlight in stoppered bottles for several weeks. Their "activity" was tested from time to time by adding a few drops to 5 cc. of a dilute solution of urochrome which had been isolated from about 8 liters of normal human urine by the method of Garrod,<sup>10</sup> and allowing the test tube containing the mixture to stand in the hot sunlight for a few minutes and then examining the solution for absorption bands.

The aldehyde solutions first exhibited "activity" by causing the color changes in the urochrome solution. No bands were developed at this stage. The aldehyde solutions were allowed to stand in the sunlight, however, until they became "active" in their ability to produce the absorption bands.<sup>11</sup>

The action of the aldehyde preparations on alcoholic urochrome solutions was identical with that described by Garrod. The same result was obtained when a small amount of aldehyde was allowed to act upon alcoholic solutions of lactochrome, prepared either by Blyth's method or by merely extracting the ammonium sulphate saturated whey with absolute alcohol. On warming a small quantity of lactochrome solution to which about 10 per cent "active" aldehyde had been added, or allowing the same to stand in the warm bright sunlight, the yellow color of the solution deepened to an orange tint and the previously spectroscopically inert solution exhibited the absorption band of a neutral or acid urobilin solution. Ammonium hydroxide changed the color to a light yellow with the disappearance of the band. A few drops of zinc chloride solution, however, produced the broad band of "urobilin zinc," the solution at the same time showing the usual brilliant green fluorescence of such solutions. Completely in accord with the observations of Garrod, when the action of the aldehyde was

<sup>10</sup> *Proc. Roy. Soc.*, lv, p. 384, 1894.

<sup>11</sup> Aldehyde solutions retain their "activity" for a long time. The sample prepared from Merck's e.p. acetaldehyde was still very "active" after it had stood in the author's laboratory for twelve months.

allowed to continue upon the solutions of both urochrome and lactochrome the color deepened to red-brown and a second absorption band appeared in the blue about 200 wave lengths removed from the first band.

A number of photographs were made of the result of the action of "active" aldehyde upon the alcoholic solutions of urochrome and lactochrome. These are shown in the accompanying plates. The photographs were made on Cramer's spectrum plates using an Adam Hilger spectroscope<sup>12</sup> with photographic attachment. The prism and lenses were of crown glass. An 80 candle power Mazda lamp was used for the source of light, arranged in a special box so that only the spectrometer slit and the cell containing the solution were illuminated. A photograph of an electric spark in hydrogen atmosphere was made on each plate, to have a few solar lines for comparison, especially the F line.

The product that resulted when the transformation of lactochrome was allowed to proceed only to the "one band" stage resembled natural urobilin in that it was now almost completely precipitated as red flakes on saturation of the aqueous solution with ammonium sulphate. The precipitated pigment was soluble in alcohol and chloroform and could not be extracted from the latter solution with water. These solutions showed the urobilin absorption band.

"Active" aldehyde was found to have a very characteristic action upon the greenish yellow milk serum from which the casein, fat and coagulable proteins had been removed. On standing in the hot bright sunlight or gently warming on the steam bath, after the addition of about 10 per cent "active" aldehyde, small quantities of the serum which were practically colorless as viewed in the test tube and exhibited no absorption bands either before or after acidifying with HCl, took on a light rose color which gradually changed to a lavender. At the same time one and finally two absorption bands appeared. Sometimes the bands were in the normal positions and again they were considerably shifted toward the red end of the spectrum. Ammonium hydroxide destroyed the bands and changed the color to a faint pink. The cause of

<sup>12</sup> We are indebted to Dr. O. M. Stewart, Chairman of the Physics Department, University of Missouri, for the use of this instrument and assistance in making the photographs.

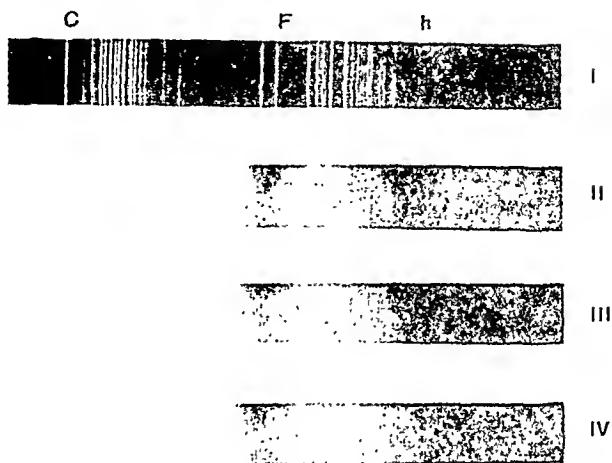


FIG. 1.

- I. Spectrum of electric spark in hydrogen.
- II. Absorption band of urobilin in acid solution.
- III. Band produced by the action of "active" aldehyde on urochrome.
- IV. Band produced by the action of "active" aldehyde on lactochrome.

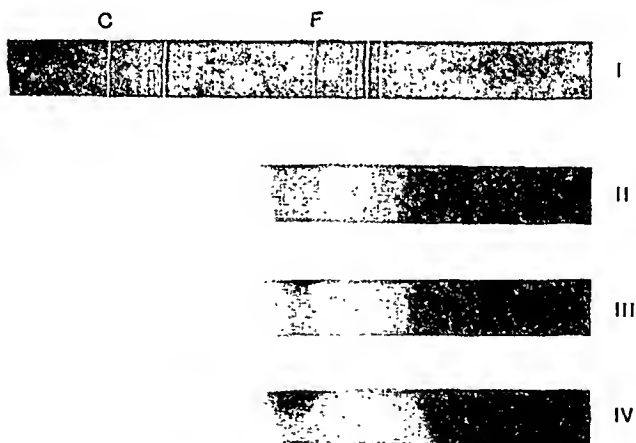


FIG. 2.

- I. Spectrum of electric spark in hydrogen.
- II. Normal absorption band of "urobilin zinc."
- III. Band produced from urochrome by aldehyde after treatment with  $\text{NH}_4\text{OH}$  and  $\text{ZnCl}_2$ .
- IV. Band produced from lactochrome by aldehyde after treatment with  $\text{NH}_4\text{OH}$  and  $\text{ZnCl}_2$ .



the shifting of the bands, which was sometimes obtained, was not investigated. It may possibly have been due to the fact that less alcohol was present in these cases on account of less aldehyde being used.

*Action of "active" aldehyde on urobilin.*

A few observations were made of the action of "active" aldehyde on urobilin. The urobilin solution was an impure one prepared by merely extracting a quantity of acidified (with HCl) urine with chloroform, evaporating the extract to dryness and dissolving in alcohol. The golden yellow solution thus obtained showed the usual urobilin absorption band. The solution had an acid reaction. The addition of aldehyde produced a strong green fluorescence like a solution of "urobilin zinc," the solution at the same time showing a broad band like that of "urobilin zinc" except that it appeared to have superimposed upon it the narrow normal urobilin band. On warming the solution for some time the broad band became uniformly intense. No second band appeared and no color change was exhibited by the solution other than the green fluorescence already noted.

These results make it clear that some substance other than urobilin is the cause of the characteristic reactions which urochrome and lactochrome give with "active" aldehyde.

*The bromine compound of lactochrome.*

Salomonsen<sup>13</sup> and especially Mancini<sup>14</sup> have recently discovered that urochrome forms a very characteristic compound with bromine. We therefore made a study of the products formed on bromination of a concentrated aqueous solution of the freshly prepared<sup>15</sup> lactochrome from 4 liters of highly colored skim milk whey, with a view of further establishing the relation between lactochrome and urochrome.

<sup>13</sup> *Biochem. Zeitschr.*, xiii, p. 205, 1908.

<sup>14</sup> *Ibid.*, xiii, p. 208, 1908.

<sup>15</sup> The other details of this method of isolation as well as all other isolations of both urochrome and lactochrome may be found in Research Bulletin No. 13, Missouri Agricultural Experiment Station.

The immediate effect of the addition of bromine to the lactochrome solution was the precipitation of a brownish yellow granular substance. When the addition of bromine caused no more of this substance to come down the solution was set aside in the ice-box over night. In the morning the precipitate was filtered off on a hardened filter paper and thoroughly washed with water in which it was insoluble.

*The precipitate.* The precipitate after drying had a light brownish yellow color and a granular appearance. In many respects it closely resembled the bromine compound of urochrome as described by Salomonsen and Mancini. It was slightly soluble in hot water and hot absolute alcohol, but insoluble in the cold reagents. It was, however, readily soluble in both water and alcohol in the presence a very little alkali ( $\text{NaOH}$ ,  $\text{KOH}$ , or  $\text{NH}_4\text{OH}$ ), giving yellow to brown solutions. The compound was precipitated from its slightly alkaline solutions as a green precipitate by  $\text{CuSO}_4$ , as a red precipitate by  $\text{FeCl}_3$ , and as a faintly yellow precipitate by  $\text{AgNO}_3$ . The silver salt was readily soluble in an excess of  $\text{NH}_4\text{OH}$ , and was reprecipitated by  $\text{HNO}_3$ . The latter precipitate became granular on heating and darkened to a brownish color in the sunlight.

The substance gave a strong flame test for halogen but did not give up its bromine on heating with strong  $\text{KOH}$ . The substance gave a strong pyrrol reaction to a pine splinter moistened with  $\text{HCl}$  when heated with zinc dust.

Contrary to the bromine compound of urochrome the substance was not readily soluble in glacial acetic acid or 60 per cent alcohol. It was slightly soluble in boiling glacial acetic acid and was reprecipitated from the solution, after cooling, on the addition of ether.

Alcoholic solutions of the bromine compound obtained with the aid of a little alkali and carefully neutralized with  $\text{HCl}$  showed the interesting property of reacting with "active" aldehyde, like solutions of free urochrome and lactochrome. When placed in the hot sunlight the light yellow color of the solutions turned to a dark brownish red and showed the absorption band of urobilin. Ammoniacal zinc chloride changed the color to a golden yellow, the solution now showing the usual green fluorescence and broad absorption band of "urobilin zinc."

*The filtrate.* The filtrate which remained after bromination of the

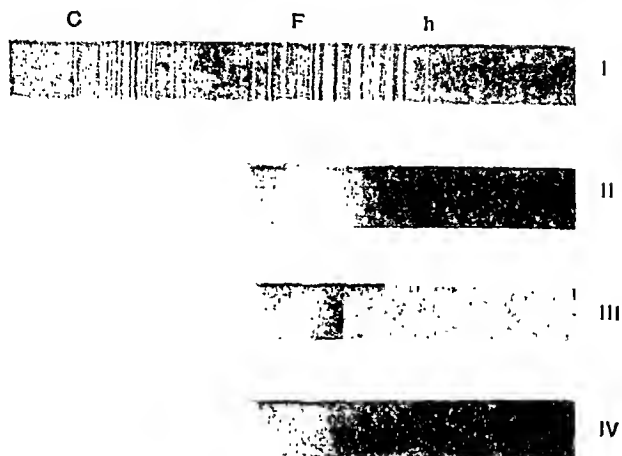


FIG. 3.

I. Hydrogen spectrum.

II. Absorption band produced by action of aldehyde on urochrome.

III and IV. Two different exposures of a solution of urochrome in which the reaction with aldehyde had been allowed to develop to the second band stage. Both bands are clearly seen.

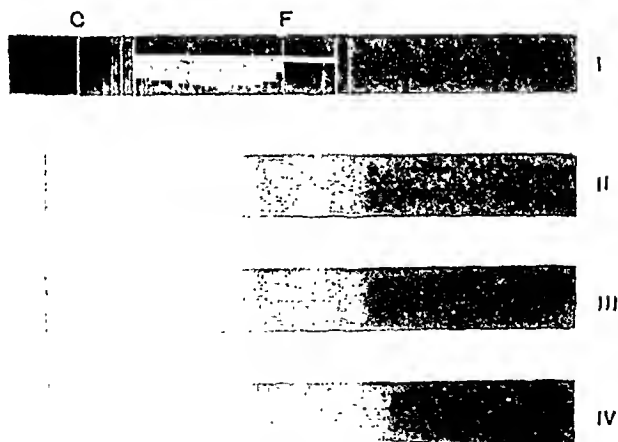


FIG. 4.

I. Hydrogen spectrum.

II, III and IV. Three exposures of a lactochrome solution showing the development of the second band.



lactochrome solution did not give up its deep red bromine color on heating or in fact on evaporation to dryness, but left instead a heavy red colored residue several times the weight of the bromine compound which had been precipitated. This residue was readily divided into two portions, one soluble in absolute alcohol and the other insoluble. The insoluble portion had a flaky appearance and chocolate brown color. It was readily soluble in water or dilute alcohol giving solutions of a dull brownish color. The absolute alcohol-soluble portion left on evaporation a reddish brown, gummy residue. Its solutions had a brilliant golden yellow color.

Both portions of the original residue were rich in loosely combined bromine, readily giving it up in alkaline solution so that on neutralization it was thrown down by silver nitrate, the white granular precipitate in both cases rapidly turning purple in the sunlight. Neither portion of the original residue gave a pyrrhol reaction on heating with zinc dust.

Alcoholic solutions of the chocolate brown residue gave a strong reaction with "active" aldehyde, quickly turning to a rose to a deep red color in the sunlight and showing the urobilin band and other properties of urobilin. Solutions of the gummy residue also gave the reaction with aldehyde but not so clearly and with much greater difficulty. This was probably due to the presence of small amounts of the other residue taken up by long contact with the absolute alcohol.

#### *Lactochrome in sheep's milk and human milk.*

Opportunity was afforded to observe the whey from a number of samples of sheeps' milk. Some of the samples were characterized by a very much higher color than any of the many samples of whey of cows' milk examined by us.<sup>16</sup> The pigment was found to be identical in every respect with the lactochrome of cows' milk. "Active" aldehyde when acting on alcoholic extracts of the ammonium-sulphate saturated whey produced the urobilin-like pigment and its accompanying properties in even more striking manner than when acting on similar solutions from cows' milk. The development of the second absorption band was especially pronounced.

<sup>16</sup> One sample gave a colorimetric reading of 24 units of yellow. See colorimetric readings of cows' milk for comparison.

One sample of human milk was examined for the presence of lactochrome, with the result that a small quantity of a yellow alcohol-soluble pigment was isolated which gave the reaction with "active" aldehyde. On account of the well known difficulty of obtaining an acid coagulation of the casein of human milk the lactochrome was isolated as follows: The milk (volume about 350 cc.) was acidified with acetic acid, a little NaCl and  $(\text{NH}_4)_2\text{SO}_4$  added and the milk boiled for a few minutes. The precipitate was filtered<sup>17</sup> off and washed with a little 95 per cent alcohol. The extract was evaporated to dryness at a low temperature and freed from fat with ether. The ether insoluble residue dissolved readily in alcohol with a yellow color. It was this solution that gave the reaction with "active" aldehyde, turning to a deep red color in the warm sunlight and showing the properties of urobilin, including the green fluorescence and broader band after treatment with  $\text{NH}_4\text{OH}$  and  $\text{ZnCl}_2$ .

*Some factors influencing the color of the whey of cows' milk.*

A wide variation in the color of the whey from the milk of different cows was observed during the course of our investigations. A study was made of some factors that might influence the amount of lactochrome in the whey. A colorimetric reading of the whey from a large number of cows was made by preparing a sample of perfectly clear whey in a uniform manner and observing its color at a depth of 10 cm. using the Lovibond tintometer and standard color glasses.

Color readings were made of the whey from the milk of forty-three different cows and six sheep. The cows were all pure bred dairy animals and represented four breeds: four Ayrshires, four Shorthorns, fifteen Holsteins and twenty Jerseys. Their milk production varied from 4.2 to 47.4 pounds per day, the stage of their lactation period from one to thirteen months, and their ages from three to fifteen years. The cows were all receiving the same ration.

By arranging the colorimetric readings according to the breed, stage of lactation, age and volume of milk production it was clearly shown that the color of the whey is primarily a breed character-

<sup>17</sup> The filtrate was colorless.

istic, the Ayrshire and Jersey breeds giving appreciably higher colored milk wheys than the Holstein and Shorthorn. This became apparent while we were taking the colorimetric readings. In almost every case it was possible to select the Ayrshire and Jersey samples by mere observation of the color of the cloudy yellow whey which remained after the removal of the casein. The average colorimetric readings for the different breeds were as follows:

	Units of yellow
Ayrshire.....	4.78
Jersey.....	3.49
Holstein.....	2.41
Shorthorn.....	2.15

The stage of lactation and the age of the animals had no uniform influence on the color of the whey. The volume of the milk production also did not seem to be a factor. When comparing the average color reading and milk production of the Jerseys with the average color reading and milk production of the Holsteins, the lowest color apparently went with the highest milk production. The same did not hold good, however, when comparing either the Jerseys or Holsteins with the Ayrshires. Similarly the relation of high color and low milk production did not hold good when comparing either the Jerseys or Holsteins among themselves. Many of the highest colored wheys were accompanied by a high volume of milk production, and vice versa.

#### SUMMARY AND CONCLUSIONS.

The facts brought out by this investigation point very clearly to a very close relationship existing between the yellow lactochrome of milk whey and the urochrome of urine. The general characteristics of the two pigments are identical. They are precipitated by the same reagents, and in common are not precipitated to any extent on saturation of their aqueous solutions with ammonium sulphate but can be readily extracted from the latter solutions by alcohol. The pigments show the same solubilities, and their solutions are spectroscopically inert. Either crude or fairly pure alcoholic solutions of both pigments give the same striking reaction with "active" acetaldehyde, being transformed thereby into a



## ABSORPTION AND FATE OF TIN IN THE BODY.

By WILLIAM SALANT, J. B. RIEGER AND E. L. P. TREUTHARDT.

(From the Pharmacological Laboratory, Bureau of Chemistry, U. S. Department of Agriculture, Washington, D. C.)

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Notwithstanding the hygienic importance of the presence of tin in many food products, our knowledge of its pharmacological action is extremely meager. Scattered through the literature may be found a few reports of cases of poisoning by the ingestion of tin with canned foods, or by the accidental swallowing of its soluble salts. Its toxicity has also been established by experiments on animals by Orfila,<sup>1</sup> and more recently by White,<sup>2</sup> and Ungar and Bodländer.<sup>3</sup> Attempts have likewise been made to study its absorption and elimination, but these have been so limited in extent that our knowledge of the subject is quite rudimentary. Neither the conditions of absorption nor its fate after it reaches the circulation has as yet been the subject of serious inquiry.

White was the first to study the fate of tin in the body. According to his analyses the urine collected during a period of twelve hours after the intravenous injection of tin salts contained appreciable quantities of the metal, the blood giving a precipitate with  $H_2S$  in one rabbit. But he failed to corroborate this finding. Four to five days after injection tin was found by him in the muscles, the liver and in the brain. The latter contained only 1-1.5 mgms. Ungar and Bodländer reported experiments on one rabbit and one dog. Tin which was given subcutaneously was eliminated in the urine and in the feces, by far the greater part being passed in the urine. The liver contained considerable quantities; much smaller amounts were present in the muscles. The distribution of this metal in the body is therefore quite general

<sup>1</sup> Orfila: *Traité des Poisons*, Paris, 1818, p. 352.

<sup>2</sup> White: *Arch. f. exp. Path. u. Pharm.*, xiii, p. 53, 1880.

<sup>3</sup> Ungar and Bodländer: *Zeitschr. f. Hyg.*, ii, p. 241, 1887.

according to the work of previous investigators. In a recent paper on the action of the heavy metals, Moore, Oldershaw and Williams<sup>4</sup> maintain that elimination takes place in various parts of the intestinal canal but no quantitative data were published.

Our report deals chiefly with the elimination of the metal by the kidneys and the gastro-intestinal canal after it was administered subcutaneously to normal animals, special attention being paid to securing information regarding the rate of elimination. The amounts of tin in the urine and feces were therefore examined at intervals of one or more days after its administration. Experiments were also carried out to test whether or not absorption takes place when the salts are given by mouth. The distribution of the metal when administered by various methods was studied in a number of animals but a more detailed study of this subject together with investigations on the absorption and elimination of tin under pathological conditions is in progress.

### *Method.*

Rabbits, cats, dogs and rats were the subjects of our experiments. Tin in the form of sodium stannous tartrate, or tin tartrate, was administered subcutaneously, intravenously and by mouth. The urine and feces were collected and examined separately for tin. It was also determined in a number of cases in the contents of the stomach and intestines, in the blood, liver and skin. The gravimetric, as well as the volumetric, method was employed, the following being the mode of procedure:

The material was digested with concentrated nitric acid until it became semi-fluid. Sulphuric acid was added and the mass was heated until charred. After cooling, nitric acid was again added and digestion continued until a clear solution was obtained free from nitric acid. The digestion had to be modified according to the particular substance to be analyzed. On account of the large amount of substance the skin was cut into three or four portions, which were united before precipitation with hydrogen sulphide. The intestinal contents were evaporated with the addition of nitric acid to a pasty mass. This was then neutralized with ammonia, made slightly acid, and hydrogen sulphide passed through it. The stannic sulphide thus formed was filtered off through asbestos in the case of the volumetric method and through paper for gravimetric determinations. As copper is frequently

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<sup>4</sup> Moore, Oldershaw and Williams: *Brit. Med. Journ.*, ii, p. 217, 1913.

present in animal organs the stannic sulphide was dissolved in potassium hydroxide instead of ammonium polysulphide. It was found later, however, that the use of ammonium polysulphide is more convenient and the results just as accurate. The tin was then precipitated with acetic acid, the precipitate dried, ignited and weighed as stannic oxide.

In some of the experiments the following method was used:

The material was treated with  $\text{H}_2\text{SO}_4$  and  $\text{HNO}_3$  and ignited in the muffle at a low red heat. The ash was leached with dilute  $\text{HCl}$ , the insoluble portion fused with  $\text{Na}_2\text{CO}_3\text{--K}_2\text{CO}_3$  in a porcelain crucible and added to the main portion. The tin was precipitated as  $\text{SnS}_2$ , dissolved in  $(\text{NH}_4)_2\text{S}$ , reprecipitated with acetic acid, filtered on a tared Gooch crucible and carefully ignited to  $\text{SnO}_2$ . About 10 cc. of concentrated  $\text{HNO}_3$  were then run through, followed by a few cc. of  $\text{H}_2\text{O}$ , the crucible ignited and weighed. The  $\text{SnO}_2$  was reduced on charcoal with a blow pipe, and the resultant globule of tin identified with a magnifying glass. Amounts of  $\text{SnO}_2$ , less than 5 mgms., may be dissolved in concentrated  $\text{H}_2\text{SO}_4$  and deposited on the gold wire of a gold-zinc wire couple. For the volumetric determination the procedure recommended by Baker<sup>5</sup> was followed. In the presence of a large amount of insoluble residue it is necessary, when using this method, to decant the hydrochloric acid solution from the residue after boiling, and reextract a couple of times with fresh hydrochloric acid.

#### EXPERIMENTAL.

**SERIES I.** Twenty-five milligrams of tin per kilo in the form of the double salt were given by subcutaneous injection to four rabbits, the urine being collected by exerting pressure on the bladder through the abdominal wall. As will be noticed by inspecting Table I, the elimination of tin was appreciable several hours after the administration, but the amounts excreted during much longer periods were not in proportion when compared with that found in the urine of Rabbit 981, notwithstanding the greater amounts of urine passed in these periods. The reaction of the urine in Experiments 1 and 2, it will be noticed, was neutral or alkaline, an acid reaction being observed at times only in Experiment 2, while in Rabbit 981 acid urine only was obtained. The solvent action of acids on tin may perhaps be responsible for the increased rate of elimination of tin in the case of Rabbit 981.

<sup>5</sup> Baker: *Eighth International Congress of Applied Chemistry*, xviii, No. 7, p. 35, 1912.

TABLE I.

EXP. NO.	RABBIT NO.	DURATION	URINE	REACTION	TIN PRESENT
		<i>hours</i>	<i>cc.</i>		<i>mgms.</i>
1	982 986	24	47	neutral and alkaline	3.5
2	985 986A	48	172	neutral, acid, alkaline	3.0
3	981	6.5	11	acid	2.0

SERIES II. Three rabbits 997, 998, 1000, weighing 2205, 2285 and 2025 grams, respectively, received by subcutaneous injection 25 mgms. of tin per kilo, as tin tartrate. Twenty-four-hour composite samples of urine and feces were analyzed for tin as shown in Table II. The daily amounts present in the urine, it will be noticed, exceed those present in the feces. Though relatively very large, the difference in the absolute amounts is not very striking. The comparatively small amounts eliminated by the kidneys and gastro-intestinal canal—not quite 10 mgms. of tin at the end of the first day and smaller amounts on subsequent days—indicates that the excretion or absorption, or both, is very slow. Similar results were obtained in the next series of experiments.

TABLE II.

DATE FEB.	URINE	REACTION	TIN PRESENT		
			Urine	Feces	Total
	<i>cc.</i>		<i>mgms.</i>	<i>mgms.</i>	<i>mgms.</i>
7	195	acid	5.4	2.9	8.2
8	170	acid	4.0	2.0	6.0
9,10	145	acid	1.3	1.3	2.5
11	220	acid	4.2	1.1	5.3

SERIES III. Diet, oats and cabbage. Three rabbits Nos. 1023, 1024 and 1025, weighing 2055, 2030, 1385 grams, respectively, received by subcutaneous injection 25 mgms. of tin per kilo in the form of stannous tartrate. The urine and feces were collected and in each case composite samples analyzed.

TABLE III.

EXP. NO.	DURATION	URINE	TIN PRESENT		
			Urine	Feces	Total
	<i>days</i>	<i>cc.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>mgms.</i>
1	2	525	9.7	4.8	14.4
2	4	1050	3.2	5.1	8.3
3	4	650	0.8	2.3	3.1

The entire amount recovered was 32.6 mgms. of  $\text{SnO}_2$  or 25.7 mgms. of tin, which is 18.75 per cent of the amount injected into the three rabbits. As in the preceding experiment elimination was quite marked at the beginning of the experiment and seems to slow down progressively during the succeeding periods. The amount present in the feces in the second period was greater than in the urine in this, as well as in the next period, thus indicating a greater excretory action on the part of the gut.

SERIES IV. Three rabbits, Nos. 1031, 1032 and 1033, diet cabbage and oats, weighing 1620, 1930 and 1430 grams, respectively, received subcutaneously 25 mgms. of tin per kilo as tin tartrate, or a total of 99.2 mgms. of tin. Composite samples of urine and feces were collected and examined separately for tin as in previous experiments.

TABLE IV.

TIME	URINE	TIN PRESENT		
		Urine	Feces	Total
	<i>cc.</i>	<i>mgms.</i>	<i>mgms.</i>	<i>mgms.</i>
1st day.....	500	9.2	9.8	19.0
2d-8th days.....	1250	7.4	3.3	10.8
Total.....	1750	16.7	13.1	29.8

The data presented in this table again show that the largest amount of tin was eliminated on the first day of the experiment, the rate of elimination in the urine and feces being the same. The rôle which diuresis plays in the excretion of tin is rather uncertain, but the evidence presented in the experiments cited thus

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far makes it extremely doubtful whether diuresis is a factor in the excretion of tin by the kidneys.

Since the tin in the feces may represent only a portion of that which was excreted into the lumen of the alimentary canal, experiments were carried out in which were determined the amounts present in the feces, gastro-intestinal contents, as well as in the walls of the stomach and intestines. Fifty milligrams of tin were given subcutaneously to each of the rabbits, which were killed forty-eight hours later. Elimination in the urine and the distribution in some of the organs were also studied. The results are presented in Table V. As in the other experiments presented above, the urine, with one exception, contained more tin than the feces. The total amount, however, excreted by the gastro-intestinal canal of each rabbit was appreciably greater than was found in the urine of the same period. Thus in Rabbit 1039 it was about 5 mgms. more than in the urine; in 1038 it was more than 10 mgms.

TABLE V.

RABBIT NO.	URINE CC.	MILLIGRAMS OF TIN PRESENT IN									
		Urine	Feces	Intestinal contents	Stomach contents	Intestinal wall	Stomach wall	Skin	Liver	Kidneys	Rest of body
1038	25	0.4	5.1	3.6	2.1			6.0*	5.4	2.3	
1039	66	7.3	2.7	8.7	0.8	2.1	2.4	6.8†	2.8	1.6	
993	104	7.6		4.0	3.0		3.6	13.2	2.8	4.1	43.7
1043	85	6.3		0.7	0.3		1.2	9.5	0.3	0.8	47.6

\* Portion weighing 57 grams without hair.

† Portion weighing 40 grams without hair.

TABLE VI.

RABBIT NO.	MILLIGRAMS OF TIN PRESENT IN	
	Urine	Liver
981	2.0	3.7
940		8.0
946		5.0
1047	1.8	2.9
1048	4.8	1.5

In No. 1043 small amounts only were found in the feces, gastrointestinal contents and in the walls of the stomach and intestines, but this was apparently an exceptional case. The average amount of the four experiments shows, however, that the rate of elimination into the stomach and intestines was greater than into the urine.

A study of the distribution in the various organs was also made. The skin, it will be noticed, contained approximately 26 and 19 per cent of the amount injected into Rabbits 993 and 1043 respectively. In the other two rabbits it was considerably greater since only 57 grams and 40 grams of skin were taken for analysis—which constituted about one-third of the entire skin of each rabbit. The amounts of tin found in the other organs and rest of the body show that its distribution must be very general. The presence of tin in the skin may be due to its accumulation after leaving the circulation or to failure of absorption. This subject is now under investigation. Data thus far obtained show that after the intravenous injection of soluble tin compounds small quantities were found in the skin. Analysis of the livers of rabbits which received tin by subcutaneous injection in amounts varying between 25 and 46 mgms. of tin per kilo show that comparatively small quantities are deposited in this organ. Reference to Tables V and VI show that 8 mgms. of tin were found in one case, 5 mgms. were recovered in each of two others, and 1.5 to 3.7 mgms. or an average of 2.3, in six others, which was about 5 per cent of the amounts injected. The blood would naturally suggest itself as one of the tissues which might contain a good deal of the metal. But previous investigators failed to detect any tin in the blood forty-eight hours after injection. This was reported by White<sup>6</sup> and later by Ungar and Bodländer.<sup>7</sup> We carried out the following test to ascertain how long tin salts remained in the circulation. As shown in the following experiments large amounts of tin salts injected intravenously may disappear within a few hours.

Cat No. 170. Weight, 2750 grams. November 8, 2.00 p.m., 5 cc. of sodium tin tartrate containing 112 mgms. of tin injected intravenously. 4.30

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<sup>6</sup> *Loc. cit.*

<sup>7</sup> *Loc. cit.*

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p.m., cat bled to death. 60 cc. of blood obtained contained 2.1 mgms. of tin.

Dog No. 158. Blood was drawn from a dog three hours after the intravenous injection of about 200 mgms. of tin. 100 cc. of blood contained 7.6 mgms. of tin. As this amount of blood was about one-fourth of the blood content of the dog, the total blood in the dog would have contained about 30.4 mgms. of tin.

Cat No. 153. Two hours after injection of 71 mgms. of tin, 80 cc. of blood were drawn. This contained 2.3 mgms. of tin. As this amount of blood represented about four-fifths of the total in the cat, the entire amount in the blood of the cat at this time was about 2.9 mgms.

Tin in the form of the double salts, the tartrate, or the citrate, was also given by mouth. Although large doses, 46 mgms. in the form of the double salt, were fed daily for three days, the combined urine of three rabbits, collected for periods of three to four days, contained only 1-3 mgms. of tin. The livers were free from tin.

Experiments on dogs, one receiving by mouth 100-300 mgms. of tin as tin tartrate daily for four weeks, the other for two weeks, failed to show evidence of the absorption of tin from the gastro-intestinal canal. The urine which was voided spontaneously was collected for several days and examined for tin. Only small quantities were detected. When the urine was obtained by catheter the amounts of tin present were negligible—0.7 in one animal and 1.7 mgms. in twenty-four hours in another. In White's experiment a dog received by mouth increasing doses of sodium stannous tartrate for twenty-two days in amounts varying between 20 and 60 mgms. The urine collected during eight days contained 20 mgms. of tin. Experiments which we have carried out on rats that received sodium tin tartrate with their food daily for three to four months, show that tin was absorbed from the gastro-intestinal canals of these animals. It is quite possible that tin salts when fed for a considerable period of time may cause changes in the gastro-intestinal mucosa which favor absorption of the metal. This is illustrated in the following protocols:

The rats were on a diet containing increasing amounts of tin salts. The entire bodies, minus the intestinal tracts were analyzed for tin.

Rat No. 4. Body minus intestinal tract contained 2.8 mgms. of tin.

Rat No. 5. Body minus intestinal tract contained 6.2 mgms. of tin.

Buchanan and Schryver's<sup>8</sup> observations are of interest in this connection. Examination of the urine of dogs which received daily large amounts of sodium stannous tartrate showed the presence of tin in the urine of the second and third week of the experiment, but none was found in the urine of the first week. This demonstrates that absorption may take place from the gastrointestinal canal when the period of feeding is prolonged.

#### RÉSUMÉ AND CONCLUSIONS.

1. After the subcutaneous injection of soluble tin salts the metal may be found in the urine and in the contents of the gastrointestinal canal, smaller quantities being present in the urine. Twenty to 25 per cent was found in the skin; the liver contained amounts varying between 1.5 and 8 mgms. or an average of about 5 per cent of the amount injected. After feeding soluble salts of tin to rabbits for three to four days, and to dogs for two to four weeks, only traces could be detected in the urine. After feeding soluble tin salts to rats for four months appreciable quantities were found in the body. Soluble tin salts given intravenously disappeared from the circulation within two to three hours.

The above data justify the conclusions that the gastro-intestinal tract is the chief organ for the elimination of tin; that the kidney plays a subordinate though an important rôle. Elimination of the metal is very slow; appreciable quantities are eliminated during the first and second days.

Absorption of tin from the gastro-intestinal tract may take place under certain conditions.

<sup>8</sup> Buchanan and Schryver: *Local Government Board (Med. Dept.), Report of Inspector of Foods*, London, 1903, p. 18.



## SOME LIMITATIONS OF THE KJELDAHL METHOD.

BY H. D. DAKIN AND H. W. DUDLEY.

(From the Herter Laboratory, New York.)

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Since the introduction, thirty years ago, of the Kjeldahl method for the determination of nitrogen, the usefulness and general employment of the process has steadily increased. It has been often said that the utility of a method is great only when its limitations are clearly determined. It is generally known that while the vast majority of organic substances containing nitrogen may be accurately analyzed by the Kjeldahl method, certain compounds yield fallacious results. Some, but not all, of the latter substances, *e.g.*, certain nitro compounds and hydrazines, may be analyzed successfully when subjected to reduction with either zinc or tin prior to decomposition with sulphuric acid. The general impression as to the applicability of the Kjeldahl method may be fairly judged by the following quotation from the article by P. Rona in Abderhalden's *Handbuch der biochemischen Arbeitsmethoden*:

Der Stickstoff kann nach den Untersuchungen von F. W. Dafert ohne Vorbereitung mittelst der Kjeldahlschen Methode bestimmt werden in allen Amiden, Ammoniumbasen, Pyridin- und Chinolinkörpern, Alkaloiden, Bitterstoffen, Eiweisskörpern und verwandten Substanzen, höchstwahrscheinlich auch bei Indolabkömmlingen. Einer Vorbehandlung, die in dem Zusatz einer organischen Substanz, Reduktion mit Zinkstaub besteht, bedürfen im allgemeinen solche Körper, die Stickstoff an Sauerstoff oder an ein zweites Stickstoffatom gebunden enthalten, das sind (mit einzelnen Ausnahmen) folgende Verbindungen: alle Nitro-, Nitroso-, Azo-, Diazo-, Hydrazo-, Aminoazokörper, die Verbindungen der Salpetersäure und der salpetrigen Säure, die Hydrazine, wahrscheinlich auch die Cyanverbindungen.

We find, however, that it is unsafe to assume that the Kjeldahl method as commonly employed may be applied without modifi-

eation for the analysis of some of the substances which are placed in the first group of Dafert's classification.

We were led to doubt the applicability of the ordinary Kjeldahl method for certain cyclic compounds in the course of an experiment on the distribution of the piperidine of diethoxyacetic acid between ether and water. On analyzing the ether and aqueous solutions by heating with sulphuric acid, potassium sulphate and copper sulphate for half an hour after the solution had become perfectly clear, we found in both solutions only about a fifth of the total nitrogen actually present. We were thus led to test the method on piperidine itself, and a number of other cyclic nitrogen compounds.

Piperidine itself is extremely stable towards hot sulphuric acid and at no time undergoes extensive charring. It is known that on heating with sulphuric acid to  $300^{\circ}$ , piperidine yields pyridine and in many analyses the smell of the latter substance was apparent in the distillate. The pyridine thus formed is only slowly decomposed, as will be seen later. The difficulty of decomposing pyridine and piperidine was recognized by Asbóth<sup>1</sup> and he, unlike Dafert, regarded the Kjeldahl method as inapplicable to these substances. Martin Krüger,<sup>2</sup> on the other hand, introduced the use of potassium bichromate in the Kjeldahl method, and so obtained satisfactory values for piperine and pyridine betaine hydrochloride. This method seems not to have been widely adopted.

Our own experiments show that small amounts of piperidine may be analyzed by the Kjeldahl method provided the heating is very prolonged. For example, 0.1–0.15 gram of piperidine heated strongly with sulphuric acid (15 cc.), potassium sulphate (9 grams) and copper sulphate (0.3 gram) for half an hour gave 45 per cent of the total nitrogen in the form of ammonia; after three hours it gave 98 per cent and the theoretical amount after four hours' heating. With pyridine, the results were less satisfactory, for after three hours' heating, we only obtained 70 per cent of the total nitrogen. The use of mercuric oxide, potassium permanganate, or zinc, gave no better results.

The results were so unsatisfactory that we decided to make a number of analyses with cyclic compounds containing nitrogen

<sup>1</sup> *Chem. Centralb.*, xvii, p. 161, 1886.

<sup>2</sup> *Chem. Ber.*, xxvii, p. 609, 1894.

in the ring. The experiments were made with small quantities (0.15 gram) of pure compounds and the heating with sulphuric acid, copper sulphate and potassium sulphate was prolonged for at least half an hour after all signs of charring had disappeared. In many cases where low results were at first obtained the time of heating was prolonged with advantage. Our results are contained in the following table:

	SUBSTANCE	TIME OF HEATING AFTER COMPLETE CLEARING	PER CENT NITROGEN	
			Theory	Found
		<i>hours</i>		
Pyrrole derivatives	Pyrrole.....	0.5	20.9	20.7
	Indole.....	0.5	12.0	11.6
	Indole.....	2.0	12.0	12.0
	$\alpha$ -Methyl indole.....	0.5	10.7	10.2
	$\alpha$ -Methyl indole.....	2.0	10.7	10.5
	Skatole.....	0.5	10.7	8.5
	Skatole.....	2.0	10.7	10.8
Pyridine derivatives	Pyridine.....	0.5	17.7	4.2-8.3
	Pyridine.....	3.0	17.7	12.0
	$\alpha$ -Picoline.....	0.5	15.0	2.4
	$\alpha$ -Picoline.....	4.0	15.0	7.1
	Lutidine.....	0.5	13.1	10.8
	Lutidine.....	4.0	13.1	12.6
	Collidine.....	0.5	11.6	8.5
	Collidine.....	4.0	11.6	7.7
	Nicotine.....	0.5	17.3	13.3
	Nicotine.....	4.0	17.3	17.4
Piperidine derivatives	Piperidine.....	0.5	16.5	5.7-7.4
	Piperidine.....	3.0	16.5	16.2
	Piperidine.....	4.0	16.5	16.5
	Conine.....	0.5	11.0	6.2
	Conine.....	4.0	11.0	10.4
	Piperine.....	0.5	4.9	2.0-2.6
	Piperine.....	3.0	4.9	5.0
Quinoline derivatives	Quinoline.....	0.5	10.8	6.4
	Quinoline.....	4.0	10.8	10.8
	Isoquinoline.....	0.5	10.8	3.6
	Isoquinoline.....	4.0	10.8	10.3
	Quinaldine.....	0.5	9.8	6.1

	SUBSTANCE	TIME OF HEATING AFTER COMPLETE CLEARING	PER CENT NITROGEN	
			Theory	Found
Quinoline derivatives		hours		
	Quinaldine.....	4.0	9.8	7.3
	Kynurenic acid .....	0.5	7.4	6.1
	Kynurenic acid .....	2.0	7.4	7.5
	Quinine.....	0.5	7.4	6.1-6.5
	Quinine.....	1.5	7.4	7.7
	Cinchonine.....	0.5	9.5	8.0-8.6
Pyrazole derivatives	Cinchonine.....	1.5	9.5	9.7
	Pyrazolone.....	0.5	33.3	1.2-2.3
	3-Phenylpyrazolone*...	0.5	17.5	2.0
	3-Phenylpyrazolone....	2.5	17.5	11.2
	1-Phenyl-3-methyl-pyrazolone*.....	0.5	16.1	11.5-11.7
	1-Phenyl-3-methyl-pyrazolone.....	2.5	16.1	12.8
	1-Phenyl-3-acetic acid 5-pyrazolone.....	0.5	12.9	9.0
	1-o-Tolyl-3,4-dimethyl-5-pyrazolone*.....	0.5	13.8	6.2
	1-o-Tolyl-3,4-dimethyl-5-pyrazolone.....	2.0	13.8	3.5
	Dimethylamidophenyl-dimethylpyrazolon...	0.5	18.2	14.5
	1-Phenyl-3-methyl-5-chlorpyrazole.....	0.5	14.6	10.8
	1-o-Tolyl-3,4-dimethyl-5-chlorpyrazole.....	0.5	12.7	3.0
Other substances	Aeridine.....	0.5	7.8	6.0
	Aeridine.....	2.0	7.8	7.9
	Diphenylpiperazine....	0.5	11.8	11.7
	Quinoxaline.....	0.5	21.6	21.8
	$\mu$ - $\alpha$ -dimethyl-oxy-pyrimidine.....	0.5	22.6	22.3

\*Wheeler and Jamieson's zinc method.

An extended discussion of the above results is unnecessary, but the following inferences appear warranted:

I. Pyrrole and its derivatives indole, skatole and  $\alpha$ -methylin-

dole present no difficulties when analyzed by the Kjeldahl method, but it is necessary to heat for at least one hour after all signs of charring have disappeared.

II. Of the five pyridine derivatives analyzed, only one, nicotine, gave even approximate results, and in this case prolonged heating is necessary. The Kjeldahl method, as ordinarily applied, cannot be used as a general method for the analysis of pyridine derivatives.

III. The analyses of piperidine and certain derivatives show that accurate results can only be obtained after very prolonged heating. While the method may be employed successfully, it is evident that unusual care is necessary, for such long continued digestion is liable to result in loss of ammonia if the temperature is allowed to rise unduly.

IV. Quinoline, isoquinoline and some of its derivatives all gave low results when the digestion with sulphuric acid was carried on for only half an hour after all signs of charring had disappeared. Accurate results are obtainable, however, if the heating be prolonged.

V. None of the pyrazole derivatives gave satisfactory results, irrespective of the time of heating. The reduction method of Wheeler and Jamieson,<sup>3</sup> in which zinc is used, gave better but still unsatisfactory results. This modification has been employed successfully for the analysis of picrolonates, *i.e.*, salts of *p*-nitrophenyl-3-methyl-4-nitropyrazolone. The difficulty in the analysis of pyrazole derivatives is undoubtedly connected with the presence of two contiguous nitrogen atoms in the ring. In those compounds in which an aromatic group is attached to the nitrogen, a materially higher proportion of the nitrogen was converted into ammonia.

VI. A few other compounds, derivatives of piperazine, quinoxaline and acridine and a pyrimidine derivative gave satisfactory results, although prolonged heating was necessary in the case of acridine.

From the experience of others, as well as our own, it appears that most pyrrolidine, pyrimidine, and glyoxaline derivatives,

<sup>3</sup> This *Journal*, iv, p. 114, 1908.

including histidine and proline, may be readily analyzed without difficulty. We also find that substances containing the hydantoin nucleus present no difficulty.

The application of the above results to analytical practice is obvious and needs no further comment. It would appear very desirable that whenever the Kjeldahl method is used for the identification by analysis of cyclic nitrogen derivatives suitable control analyses with pure substances should be carried out.

## THE CHEMISTRY OF GLUCONEOGENESIS.

### VII. CONCERNING THE FATE OF PYRUVIC ACID IN METABOLISM.<sup>1</sup>

By A. I. RINGER.

(From the Department of Physiological Chemistry of the University  
of Pennsylvania, Philadelphia, Pa.)

(Received for publication, January 27, 1914.)

The fate of pyruvic acid in the animal body has been the subject of considerable discussion in the past two years. P. Mayer<sup>2</sup> administered 7 to 8 grams of pyruvic acid to normal rabbits and found that the animals, when in a good state of nutrition, developed hyperglucaemia and glucosuria, while those that had been starved, and were therefore poor in glycogen content, developed hyperglucaemia only and no glucosuria. In those experiments he found that the urine often contained albumin after pyruvic acid administration, and further found that the administration of 10 to 15 grams brought about fatal intoxication. After pyruvic acid administration, he also found lactic acid in the urine.

In a second communication,<sup>3</sup> which appeared after our work was far advanced, Mayer reported the influence of pyruvic acid on gluconeogenesis of phlorhizinized dogs and rabbits. In none of his experiments was there an increase in the glucose elimination after pyruvic acid administration, while in two experiments on dogs (out of four) there was a very remarkable reduction in the glucose and nitrogen elimination. The kidneys of the dog in experiment 10 were examined microscopically and the following pathological conditions were found: "kalkhaltige Cylinder in den geraden Kanälen der Papille, Trübung und geronnene Massen in den Tubuli contorti, Hämoglobinniederschläge." He then drew the conclusion that pyruvic acid is a toxic substance, which causes a

<sup>1</sup> Aided by a grant from the Rockefeller Institute for Medical Research.

<sup>2</sup> P. Mayer: *Biochem. Zeitschr.*, xl, p. 441, 1912.

<sup>3</sup> P. Mayer: *ibid.*, xlix, p. 486, 1913.

depression in the nitrogen and glucose elimination by decreasing the permeability of the kidneys. He offered no explanation for the failure of pyruvic acid to bring about a decrease in the permeability of the kidneys in experiments 8 and 9, in which similar amounts of pyruvic acid were given, without any appreciable change in the glucose and nitrogen eliminations. In fact, there was a slight increase in both nitrogen and glucose output in experiment 9.

In a series of experiments reported by us<sup>4</sup> it was found that pyruvic acid, when given to phlorhizinized dogs *per os* or subcutaneously was not a toxic substance, and that it was glucogenetic. It is true that the glucogenetic properties of pyruvic acid were not found to be very constant, but in no case did we get the drop in nitrogen and sugar output as was observed by Mayer. Simultaneously with our communication Dakin and Janney<sup>5</sup> reported the results of their experiments from which they came to conclusions very similar to ours. They also found that pyruvic acid was glucogenetic, and in no case did they get a drop in the nitrogen and sugar output similar to that obtained by Mayer. Results similar to Dakin and Janney's and to ours have since been reported by Cremer.<sup>6</sup>

In three different laboratories experiments with pyruvic acid showed that it was glucogenetic and non-toxic. Mayer was the only one who obtained two negative results and two results which show very plainly that his pyruvic acid contained something that was toxic and had a peculiar effect upon the kidneys, an effect which resembles in its microscopical lesion as well as in its functional disturbance, the results that Underhill<sup>7</sup> and Pearce and Ringer<sup>8</sup> obtained after tartaric acid administration to phlorhizinized and normal dogs.

In his third communication on this subject<sup>9</sup> Mayer denies the presence of tartaric acid in his pyruvic acid, and suggests that the difference in our results may be due to polymerization of our pyruvic acid during the process of neutralization.

<sup>4</sup> Ringer: this *Journal*, xv, p. 145, 1913.

<sup>5</sup> Dakin and Janney: *ibid.*, xv, p. 177, 1913.

<sup>6</sup> Cremer: *Berl. klin. Wochenschr.*, 1913, No. 31.

<sup>7</sup> Underhill: this *Journal*, xii, p. 115, 1912.

<sup>8</sup> Pearce and Ringer: *Journ. of Med. Research*, xxix, p. 57, 1913.

<sup>9</sup> P. Mayer: *Biochem. Zeitschr.*, lv, p. 1, 1913.

This explanation is not valid for two reasons: First, Dakin and Janney<sup>10</sup> have shown that polymerized pyruvic acid is non-glucogenetic. Second, we have, as Mayer has, observed great care in the process of neutralization. To eliminate all doubt, however, we performed one experiment in which pyruvic acid was administered subcutaneously unneutralized. As is seen from the record of the experiment, it possesses distinct glucogenetic properties, and has no toxic effect on the kidneys.

EXPERIMENT XXXIII. Twelve-hour periods.

DATE Oct. 1913	PERIOD	WEIGHT	NITROGEN	GLUCOSE	D:N	EXTRA GLUCOSE	ACETONE AND ACETO- ACETIC ACID	$\beta$ -HYDROXY BUTYRIC ACID	REMARKS
15	XI	11.9	6.40	24.00	3.75	4.7	0.262	1.27	8.8 gms. of pyruvic acid dissolved in 3 cc. of olive oil given subcutaneously.
15	XII		6.62	27.56	4.16		0.185	0.72	
16	XIII	11.5	6.18	25.14	4.07		0.216	0.76	

We therefore still feel convinced that Mayer's results cannot be attributed to pyruvic acid, but to some extraneous influence.

*The fate of pyruvic acid in the animal body.*

In his first communication<sup>11</sup> Mayer showed that after the administration of pyruvic acid, *dl*- and *d*-lactic acid appeared in the urine. Embden and Oppenheimer<sup>12</sup> corroborated these findings. They perfused the extirpated surviving liver of dogs with blood to which pyruvic acid as ammonium or sodium salt had been added and found an increase in the lactic acid content of the perfused blood.

In another communication<sup>13</sup> Embden and Oppenheimer report their experiments on the influence of pyruvic acid on the formation

<sup>10</sup> Dakin and Janney: *loc. cit.*

<sup>11</sup> P. Mayer: *loc. cit.*

<sup>12</sup> Embden and Oppenheimer: *Biochem. Zeitschr.*, lv, p. 337, 1913.

<sup>13</sup> Embden and Oppenheimer: *ibid.*, xlv, p. 186, 1912.

of aceto-acetic acid in the perfused surviving liver of dogs. Twelve experiments were performed. Five gave no increase in aceto-acetic acid, and seven gave a very marked increase. They concluded that *pyruvic acid possesses the power of yielding aceto-acetic acid, because of the intermediary formation of acetaldehyde, which undergoes aldol condensation.*

From all this, we see that pyruvic acid can give rise to lactic acid on the one hand and to acetaldehyde on the other. In this connection it is important to remember that from Embden's experiments it is evident that *acetaldehyde is not always formed from pyruvic acid.*

In our experiments we found that *in some instances pyruvic acid yielded large quantities of glucose and in others it gave almost negative results.* On examining the relationship between the glucose formation and antiketogenesis in our experiments, a remarkable fact is evident: in case of high sugar formation from pyruvic acid, there is a marked depression in the acidosis (experiments XXII, XXIII and XXV); conversely, when there is little sugar formation, there is practically no change in the acidosis. Ringer and Frankel<sup>14</sup> have recently shown that when acetaldehyde is administered subcutaneously to phlorhizinized dogs, it possesses the power of causing an increase in the glucose elimination and a decrease in the acidosis.

On correlating all these facts it becomes evident that *pyruvic acid possesses its glucogenetic properties because acetaldehyde and lactic acid are formed in its intermediary metabolism.*

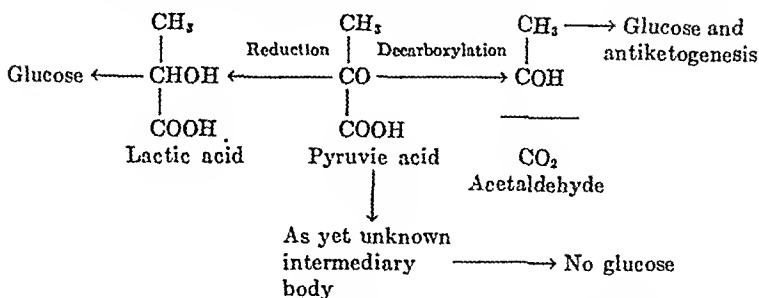
These two substances cannot possibly arise as a result of the same chemical process, and pyruvic acid must therefore be capable of following several paths of metabolism, as stated in our previous communication. The variable is not the pyruvic acid, but probably factors of equilibrium in the animal organism<sup>15</sup> and we believe there must exist a third possibility for the breakdown of pyruvic acid which results in no sugar formation. This would account for the very low sugar formation in experiments XXIV and XXVI of our series.<sup>16</sup>

<sup>14</sup> Ringer and Frankel: this *Journal*, xvi, p. 563, 1914.

<sup>15</sup> Greer, Witzemann and Woodyatt: *ibid.*, xvi, p. 455, 1914.

<sup>16</sup> Ringer: *ibid.*, xv, p. 152, 1913.

We may therefore formulate the fate of pyruvic acid in the animal organism by the following scheme.



Another contribution recently appeared, also dealing with the question of sugar formation from pyruvic acid.<sup>17</sup> Its author, Barrenscheen, perfused the extirpated liver of a phlorhizinized dog with blood to which pyruvic acid as sodium salt had been added. He found no increase in the glucose concentration of the blood after perfusion, and he therefore concluded that pyruvic acid is not a glucogenetic substance.

From what was said above it becomes evident that the method of experimentation employed by Barrenscheen, is not at all adapted for settling this question. Since it was shown that the glucogenetic properties of pyruvic acid may be very largely due to the intermediary formation of acetaldehyde, and as it was also shown that in liver perfusions acetaldehyde undergoes aldol condensation with the formation of acetone bodies, whereas in the organism as a whole it causes the formation of extra glucose, the failure of the above author to find any increase in glucose in his experiment does not in any way lend support to Mayer's conclusions.<sup>18</sup>

Perfusion experiments with pyruvic acid, through the liver, may, however, become instructive if simultaneous analysis be made of the lactic acid, aceto-acetic acid and glucose concentration of the blood before and after the perfusion. This will show whether lactic acid is formed in those experiments where acetaldehyde fails to be formed, or whether the two substances are formed simultaneously.

<sup>17</sup> Barrenscheen: *Biochem. Zeitschr.*, lviii, p. 299, 1913.

<sup>18</sup> The same argument is applicable to the work of Parnas and Baer: *ibid.*, xli, p. 386, 1912.



## PREPARATION, COMPOSITION AND PROPERTIES OF CASEINATES OF MAGNESIUM.

By LUCIUS L. VAN SLYKE AND ORRIN B. WINTER.

(From the Chemical Laboratory of the New York State Agricultural  
Experiment Station, Geneva, N. Y.)

(Received for publication, January 29, 1914.)

Van Slyke and Bosworth<sup>1</sup> have published the results of a study of compounds formed by casein with the elements of several alkaline and alkaline-earth bases. In the case of calcium, for illustration, it was found that at least four caseinates could be formed: (1) mono-calcium caseinate, (2) di-calcium caseinate, (3) neutral (or penta-valent) calcium caseinate, neutral to litmus, and (4) basic (or octo-valent) calcium caseinate, neutral to phenolphthalein. It seemed desirable to undertake a similar study to ascertain whether casein forms corresponding compounds with magnesium. The details of this work are given in this article.

### *Solution of casein in magnesium hydroxide.*

In the preparation of caseinates of the alkaline earth elements the first step in the process is to obtain a solution of casein in the hydroxide. In the case of magnesium, its hydroxide is only slightly soluble in water and the solution is so dilute as to have very little effect in dissolving casein. It was found, however, that when casein is suspended in water with an excess of finely divided magnesium oxide and allowed to stand several days with occasional agitation, enough casein is taken into solution to furnish material which can be used in the preparation of magnesium caseinates.

<sup>1</sup> This *Journal*, xiv, pp. 203-236, 1913.

*Preparation and composition of mono-magnesium caseinate.*

In preparing mono-magnesium caseinate, the remaining portion of the solution of di-magnesium caseinate was treated with enough acid to precipitate three-fourths of the casein, the acid being added very slowly and with constant, vigorous agitation. The solution was filtered and the precipitated caseinate washed with water, alcohol and ether, after which it was dried for three days under reduced pressure, over sulphuric acid.

Determination of magnesium gave 0.13 per cent Mg (equal to 0.22 per cent MgO). Calculated to correspond with mono-calcium caseinate (0.22 per cent Ca or 0.31 per cent CaO) this compound should contain 0.13 per cent Mg (equal to 0.22 per cent MgO). Expressed in another form, our results show that 1 gram of casein combines with  $1.125 \times 10^{-4}$  gram equivalents of magnesium, which agrees with the theoretical value.

Mono-magnesium caseinate is insoluble in water, but soluble in 5 per cent solution of NaCl; at 65°C. it shows a tendency to form strings when drawn out.

*Valency of casein.*

In this *Journal*<sup>4</sup> VanSlyke and Bosworth have shown from their work with calcium caseinates the combining capacity of casein in the different compounds prepared and studied by them. It is interesting to make a comparison of their results with those obtained in working with magnesium. In the following table we arrange the results expressed, for the purpose of more direct comparison, in the form of gram equivalents of element per gram of casein.

DIFFERENT CASEINATES	VALENCIES SATISFIED IN EACH CASEINATE	GRAM EQUIVALENTS OF Ca $\times 10^{-4}$ PER GRAM OF CASEIN	GRAM EQUIVALENTS OF Mg $\times 10^{-4}$ PER GRAM OF CASEIN	GRAM EQUIVALENTS OF ELEMENT $\times 10^{-4}$ PER GRAM OF CASEIN CORRESPONDING TO VALENCIES GIVEN IN SECOND COLUMN
Mono.....	1	1.11	1.125	1.125
Di.....	2	2.21	2.14	2.250
Neutral.....	5	5.36	5.84	5.625
Basic.....	8	9.00	8.72	9.000

<sup>4</sup> This *Journal*, xiv, p. 227-9, 1913.

The agreement between the analytical results obtained (third and fourth columns) and those called for (fifth column) by the valencies of casein satisfied in each compound (second column) is marked; also the close agreement between the results obtained with calcium (third column) and those given by magnesium (fourth column) is satisfactory.

#### SUMMARY.

Base-free casein is dissolved in magnesium hydroxide containing finely divided magnesium oxide in suspension. This solution, treated with hydrochloric acid under specified conditions and subjected to dialysis, can be made to furnish four magnesium caseinates, as follows: 1. *Basic magnesium caseinate* contains 1.06 per cent Mg; 1 gram of casein combines with  $8.72 \times 10^{-4}$  gram equivalents of magnesium (theoretical,  $9 \times 10^{-4}$ ). Casein is octo-valent in this compound. The compound is soluble in water and its solution is neutral to phenolphthalein. 2. *Neutral magnesium caseinate*, which is neutral to litmus, contains 0.71 per cent Mg; 1 gram of casein combines with  $5.84 \times 10^{-4}$  gram equivalents of magnesium (theoretical,  $5.625 \times 10^{-4}$ ). Casein is penta-valent in this compound. 3. *Di-magnesium caseinate*, which is slightly soluble in water, contains 0.24 per cent Mg; 1 gram of casein combines with  $2.14 \times 10^{-4}$  gram equivalents of magnesium (theoretical,  $2.25 \times 10^{-4}$ ). 4. *Mono-magnesium caseinate*, which is insoluble in water, contains 0.13 per cent Mg; 1 gram of casein combines with  $1.125 \times 10^{-4}$  gram equivalents of magnesium (theoretical,  $1.125 \times 10^{-4}$ ).



## STUDIES IN CARBOHYDRATE METABOLISM.

### III. THE INFLUENCE OF HYDRAZINE UPON GLYCOGEN STORAGE IN THE ORGANISM, AND UPON BLOOD COMPOSITION.<sup>1</sup>

By FRANK P. UNDERHILL.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven, Conn.)

(Received for publication, January 30, 1914.)

In a previous communication<sup>2</sup> it has been demonstrated that suitable quantities of hydrazine salts subcutaneously administered to dogs cause a marked hypoglycaemia with an accompanying reduction in the glycogen content of the liver. An explanation for this behavior is lacking. The present investigation was planned to determine whether the diminution of sugar in the blood and the disappearance of glycogen from the liver is coincident with a corresponding increase of glycogen in the muscles. Such a transformation of carbohydrate material is an obvious possibility and before seeking a more elaborate interpretation of the phenomenon under discussion it seemed desirable to exclude the possibility mentioned. Also in order to detect some of the more apparent alterations in blood composition that might account for decreased sugar content, the solids and ash content of the blood of hydrazinized and normal dogs subjected to similar experimental conditions have been compared.

In all instances the animals had been kept upon a mixed diet previous to the experiment and they were in splendid nutritive condition. The hydrazinized dogs were killed forty-eight hours subsequent to the injection and since these animals refuse food during this length of time the dogs receiving no hydrazine were allowed to fast two days also. The sugar content of the blood and the glycogen content of the liver and muscles were determined by

<sup>1</sup> Aided by a grant from the Rockefeller Institute for Medical Research.

<sup>2</sup> Underhill: this *Journal*, x, p. 159, 1911.

## 294 Influence of Hydrazine upon Muscle Glycogen

the respective methods indicated in a previous paper.<sup>3</sup> The solid and ash contents were estimated by the well known procedures.

*A comparison of the composition of blood and of the content of glycogen in liver and muscle in hydrazinized and normal dogs.*

NUMBER OF DOG	SUBCUTANEOUS INJECTION OF HYDRAZINE SULPHATE	BLOOD			GLYCOGEN CONTENT OF LIVER EXPRESSED AS GRAMS OF DEXTROSE	GLYCOGEN CONTENT OF MUSCLES (LEG) EXPRESSED IN PERCENTAGES OF DEXTROSE
		Sugar content	Solids	Ash		
	mgm. per kilo	per cent	per cent	per cent		
2	50	0.04	24.93	0.72	trace	0.75
9	50	0.06	26.61	0.32	0	0.44
10	50	0.04	25.25	0.33	0	0
12	50	0.05	26.00	0.84	0	0.70
6	0	0.14	16.50	0.87	12.7	0.92
7	0	0.19	19.60	0.87	6.5	1.50

An inspection of the table presented will demonstrate clearly that a transference of the body carbohydrate, at least as glycogen, to the muscles will not suffice as an explanation for the disappearance of glycogen from the liver and the decreased blood-sugar content after the subcutaneous introduction of hydrazine sulphate to dogs. On the other hand, there are indications not only that the liver is depleted of its glycogen store, but that the amount of muscle glycogen may be markedly decreased and indeed at times may disappear entirely thus presenting an animal with a *minimum* of carbohydrate in its body. The data compare well with what may be obtained at times with phlorhizin except that in the case of the hydrazinized animal sugar never appears in the urine. It is also of interest to note that the solids of the blood may be abnormally high although the ash content may be distinctly subnormal. Although the number of experiments is perhaps too small to admit a positive statement it is worthy of note at least that there is more or less parallelism between the ash content of the hydrazinized dogs' blood and the percentage of glycogen in the muscles.

<sup>3</sup> Underhill: *loc. cit.*

## STUDIES IN CARBOHYDRATE METABOLISM.

### IV. DO HYDRAZINE DERIVATIVES SHOW THE TYPICAL HYDRAZINE EFFECT UPON BLOOD SUGAR CONTENT?<sup>1</sup>

By FRANK P. UNDERHILL.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven, Conn.)

(Received for publication, January 30, 1914.)

The influence of hydrazine upon carbohydrate metabolism leading as it may to either the prevention or the inhibition of pancreatic diabetes<sup>2</sup> suggested the possibility of finding some derivative of the compound mentioned which while still exhibiting the effect upon blood sugar content would nevertheless be free from a part, at least, of the great toxicity of hydrazine. In accordance with this idea a number of hydrazine derivatives were procured, but it was soon found that only a few could be employed for the purpose outlined above owing to the great insolubility of the substances. Experiments have been carried through, however, with the following compounds, methylhydrazine, phenylhydrazine, methylphenylhydrazine, diphenylhydrazine, and semicarbazide.

It may be stated at once that in no instance was there the influence upon carbohydrate metabolism of dogs that is so peculiarly associated with hydrazine itself.

#### EXPERIMENTAL.

The dosage employed was similar to that for hydrazine and in all cases the dogs starved subsequent to the administration of the drug. Since hydrazine produces its maximum effect in about forty-eight hours the blood sugar estimations in the present investigation were made after a similar lapse of time. The relative toxicity of the investigated substances was determined in rabbits.

<sup>1</sup> Aided by a grant from the Rockefeller Institute for Medical Research.

<sup>2</sup> Underhill and Fine: this *Journal*, x, p. 271, 1911.

## 296 Effect of Hydrazine Derivatives upon Blood Sugar

When it had been shown that a dose similar to the usual hydrazine dosage, *i.e.*, 50 mgm. per kilo, could be borne an experiment was carried out with the dog. All injections were made subcutaneously and the behavior of the animal closely watched.

### *Methylhydrazine*, $\text{NH}_2\text{NH}\cdot\text{CH}_3$ .

*Action upon rabbit.* At 11.00 a.m. 0.095 gram of methylhydrazine (Schuchart) was injected into a rabbit of 1900 grams (50 mgm. per kilo). No unusual symptoms were observed until 2.45 p.m. at which time the animal urinated and immediately was seized with convulsions—episthotonus—the fore legs appearing to be paralyzed. The convulsion lasted only two or three minutes, after which the rabbit lay sprawled on its belly.

- 3.00 p.m. Second convulsion.
- 3.15 Third convulsion. Hind legs seem paralyzed also.
- 3.20 Fourth convulsion.
- 3.45 Fifth convulsion. Grinds teeth. In constant tremor, makes sudden jumps, fore legs jerk spasmodically and continually. Salivation, mouth open, air hunger (?).
- 3.53 Convulsion. Urination.
- 3.55 Convulsion. Animal lies prostrate.
- 3.57 Convulsion.
- 4.10 Rabbit in constant motion—as though jumping at something.

Convulsions and constant motions continued up to 6.00 p.m. Animal was found dead the next morning. Autopsy showed nothing abnormal.

Urine voided after injection of methylhydrazine reduced Benedict's solution even in the cold solution. Since methylhydrazine shows a similar behavior it is probable that this substance or a closely related compound was rapidly eliminated by the kidneys.

A second rabbit upon receiving an injection of 25 milligrams of methylhydrazine per kilo gave no evidence of any symptoms described for the larger dose.

*Action upon the dog.* The subcutaneous injection of 0.42 gram (35 mgm. per kilo) methylhydrazine into a 12-kilo dog caused the animal to vomit and to refuse all food. Normally the dog was very active but after the injection she became very sluggish and lay quietly in the cage. No other symptoms developed. At the end

of two days blood was drawn from the carotid and the blood-sugar content was found to be 0.11 per cent. Glycogen was present in the liver to the extent of 0.25 gram expressed as dextrose. The liver did not present the appearance characteristic of hydrazine. The other principal organs seemed normal.

*Phenylhydrazine,  $\text{NH}_2\text{NHC}_6\text{H}_5$ .*

*Action upon the dog.* A dog of 10 kilos was given an injection of 50 mgm. per kilo of phenylhydrazine hydrochloride without producing any unusual symptoms beyond the appearance of a relatively large amount of methaemoglobin in the urine. On the second day subsequent to the injection blood was drawn from the carotid and was found to contain 0.16 per cent dextrose. The glycogen content of the liver amounted to 0.20 gram expressed as dextrose. Upon autopsy all organs appeared normal except the liver and spleen. The former was of a peculiar chocolate brown color. The spleen was enormously enlarged and dark purple in color.

*Methylphenylhydrazine,  $\text{NH}_2(\text{CH}_3)\text{N.C}_6\text{H}_5$ .*

*Action upon the rabbit.* The injection of 50 mgm. per kilo of methylphenylhydrazine sulphate into a rabbit of 1200 grams produced no abnormal symptoms.

When 100 mgm. per kilo of the same salt were introduced into a rabbit of 1600 grams no evidence of toxicity was observed.

*Action upon the dog.* A dog of 15 kilos received an injection of 1.5 grams (100 mgm. per kilo) of the above mentioned salt at 10.00 a.m. At 10.30 the animal lay prostrate and unable to rise. The symptoms gradually became more and more pronounced until death which occurred at 12.30, apparently from respiratory failure. No autopsy was made.

To a second dog of 4 kilos was given an injection of the same salt in the dosage of 50 mgm. per kilo. Soon after the administration of this compound the dog vomited and gave evidence of muscular weakness. These symptoms gradually disappeared and in a few hours the animal seemed normal. Two days after the injection blood was collected from the carotid. The blood-sugar content was 0.14 per cent. Glycogen in the liver amounted to 12.6 grams expressed as dextrose. All organs and tissues seemed normal.

*Diphenylhydrazine*,  $\text{NH}_2\cdot\text{N}(\text{C}_6\text{H}_5)_2$ .

*Action upon the rabbit.* The introduction of the above compound as the hydrochloride into rabbits failed to produce abnormal symptoms in doses of either 50 or 100 mgm. per kilo.

*Action upon the dog.* To a dog of 10 kilos was given an injection of the above mentioned hydrazine derivative (100 mgm. per kilo) without any subsequent sign of abnormality except that the animal lay quietly in the bottom of the cage. Two days later the dog seemed normal and was bled from the carotid. *Sugar was present in the blood to the extent of 0.19 per cent.* In the liver 6.4 grams of glycogen, expressed as dextrose, were found. All organs were normal.

*Semicarbazide*,  $\text{NH}_2\cdot\text{NH}\cdot\text{CO}\cdot\text{NH}_2$ .

*Action upon the rabbit.* A rabbit of 1300 grams received an injection of 65 mgm. of semicarbazide hydrochloride (50 mgm. per kilo). For a few hours an undue excitation was observed which gradually passed away.

In doses of 100 mgm. per kilo this salt caused the death of a rabbit of 1500 grams within twenty-four hours, without evidence of any significant symptoms preceding.

*Action upon the dog.* To a dog of 10 kilos was given an injection of 0.5 gram of semicarbazide hydrochloride (50 mgm. per kilo). No abnormal symptoms followed. Two days later the blood-sugar content amounted to 0.10 per cent. In the liver were found 4.5 grams of glycogen expressed as dextrose. All organs were normal.

## STUDIES IN CARBOHYDRATE METABOLISM.

### V. THE DISAPPEARANCE OF SUGAR FROM SOLUTIONS PERFUSED THROUGH THE HEART OF THE NORMAL RABBIT, AND OF ANIMALS SUBJECTED TO INANITION AND TO THE ACTION OF HYDRAZINE.<sup>1</sup>

By FRANK P. UNDERHILL AND A. L. PRINCE.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, and the Physiological Laboratory, Yale Medical School, New Haven, Conn.)

(Received for publication, January 30, 1914.)

Among the many suggestive explanations for the greatly diminished sugar content of the blood,<sup>2</sup> and the almost (and at times quite), complete disappearance of glycogen from the liver and muscles<sup>3</sup> of hydrazinized animals, stands prominent the possible rôle of muscle tissue. It may be assumed, for example, that the activity of certain enzymes may be greatly accelerated by hydrazine leading to increased carbohydrate combustion or other transformation. Granting such an hypothesis it is reasonable to suppose that an acceleration of enzyme activity would be more or less commonly distributed to all active muscles. As an example of active muscle tissue the heart may be selected and a study of the disappearance of sugar from a solution perfused through this organ might be expected to throw some light upon the problem under discussion. The results of such a study form the basis of the present paper.

*Methods.* The heart employed was that of the rabbit, it having been demonstrated<sup>4</sup> that hydrazine in sufficient quantities may produce the effects usually observed in dogs. The perfusions were made with Locke's solution and in a somewhat modified Locke's apparatus. Sugar estimations in the blood were carried through

<sup>1</sup> Aided by a grant from the Rockefeller Institute for Medical Research.

<sup>2</sup> Underhill: this *Journal*, x, p. 159, 1911.

<sup>3</sup> Underhill: See previous paper.

<sup>4</sup> Unpublished experiments.

by the method of Forschbach and Severin;<sup>5</sup> in the perfusion fluid the Allihn procedure was followed. In initiating an experiment the animal was rendered unconscious by a blow on the neck, bled, and the heart quickly removed, washed as free as possible from blood in dishes of sugar-free Locke's solution and then attached to the apparatus. With the above mentioned sugar-free salt solution the heart was perfused until entirely free from blood. The apparatus was then emptied and Locke's solution, containing approximately 0.1 per cent dextrose added. In all instances the heart beat rapidly and vigorously, the perfusion usually being rapid. As a rule the variation in heart beat was from 74 to 144 beats per minute and the drip varied accordingly. Unless otherwise stated all experiments lasted for a period of two hours. This period was selected in order to avoid any pronounced influence that could be attributed to bacterial contamination. To further rule out bacterial activity the entire apparatus was sterilized just before each experiment, the heart was isolated and attached under conditions as aseptic as possible and only sterilized wash and perfusion fluids were employed. After the period of perfusion the apparatus and heart were thoroughly perfused with sugar-free salt solution. The heart was weighed after being cut open, washed and dried with filter paper. Control trials with the apparatus alone demonstrated a loss of dextrose to the extent of approximately 2 mgm., an amount too small to play a rôle in the results to be considered below.

It has been determined that in general a maximum effect of hydrazine upon the blood sugar content is obtained in approximately two days. During this period the animals refuse all food. In our preliminary experiments to determine the disappearance of sugar from the perfusion fluid passed through non-hydrazinized hearts we chose as our controls animals subjected to inanition for a period of two days. The results of experiments with these rabbits served as our "normals" with which to compare those obtained with hydrazinized animals.

Experiments 1 to 11 inclusive, Table 1, show the figures obtained with our control rabbits, figures which correspond well with those of Locke<sup>6</sup> but much lower than those recorded by Wilenko.<sup>7</sup>

<sup>5</sup> Forschbach and Severin: *Arch. f. exp. Path. u. Pharm.*, lxxviii, p. 341, 1912.

<sup>6</sup> Locke: *Journ. of Physiol.*, xxxvi, p. 205, 1907-08.

<sup>7</sup> Wilenko: *Arch. f. exp. Path. u. Pharm.*, lxxi, p. 261, 1913.

TABLE 1.

*The disappearance of sugar from a dextrose solution perfused through the beating and non-beating rabbit's heart.*

NUMBER OF EXPERIMENT	WEIGHT OF HEART	TOTAL SUGAR DISAPPEARANCE	SUGAR DISAPPEARANCE PER GRAM HEART PER HOUR	REMARKS
	grams	mgm.	mgm.	
1	7.0	17.6	1.2	No food for 2 days.
2	6.2	23.2*	0.9	" " " 2 "
3	6.0	12.0†	0.7	" " " 2 "
8	6.0	12.0	1.0	" " " 2 "
11	5.0	16.0	1.6	" " " 2 "
44	3.6	10.5	1.4	" " " 2 "
33	5.2	40.2	3.8	" " " 3 "
31	4.3	18.0	2.0	" " " 4 "
34	4.6	26.7	2.9	" " " 4 "
35	4.8	21.5	2.2	" " " 6 "
32	4.4	25.8	2.9	" " " 6 "
38	5.9	26.0	2.2	" " " 6 "
36	6.2	27.9	2.2	" " " 8 "
39	3.7	17.8	2.3	" " " 8 "
24	5.3	13.0	1.3	Well fed animal from pen.
26	4.3	10.0	1.1	" " " " "
40	3.4	12.0	1.7	" " " " "
41	3.6	12.0	1.6	" " " " "
42	5.2	25.5	2.4	" " " " "
43	3.2	10.0	1.5	" " " " "
37	4.8	18.8	1.8	Well fed animal given 15 grams dextrose by mouth about 18 hours previous to experiment.

*Non-beating heart.*

45	5.5	12.0	1.0	Rabbit from pen.
48	3.8	19.0	2.4	Rabbit from pen.
46	3.8	15.0	2.1	Rabbit from pen.
47	3.9	11.0	1.4	No food for 4 days.

\* Experiment of 4 hours.

† Experiment of 3 hours.

Calculated upon the basis of per gram of heart weight it is evident that per hour from 0.7 mgm. to 1.6 mgm. of dextrose may disappear from the perfusion fluid.

TABLE 2.

*The disappearance of sugar from a dextrose solution perfused through the hydrazinized rabbit's heart.*

NUMBER OF EXPERIMENT	BLOOD SUGAR CONTENT IN PERCENTAGES											REMARKS
	NORMAL	Hours after Hydrazine Administration										
		10	15	20	25	30	35	40	45	50	55	
13	0.11	0.08										6.0 21.0 1.6 Liver not pale.
15	0.12		0.09									7.5 12.0 0.8 Liver very pale.
5	0.18		0.14			0.12						6.3 70.8* 2.8 Liver somewhat pale.
4	0.13		0.14			0.06						7.0 88.0† 3.1 Liver somewhat pale.
12	0.13		0.14			0.05						5.0 18.0 1.8 Liver very pale.
8	0.12		0.09				0.06					6.0 15.0 1.2 Liver not pale.
6	0.09		0.11				0.08					5.7 26.0 2.3 Liver not pale.
9	0.14							0.09				6.0 15.0 1.2 Liver very pale.
18	0.11							0.01		0.12		5.3 27.0 2.5 Liver slightly pale.
7	0.09				0.12					0.04		5.5 17.0 1.5 Liver slightly pale.
21	0.12	0.12										5.3 11.0 1.0 Liver normal.
23	0.16		0.13									4.0 16.0 2.0 Liver slate colored.
16	0.12			0.09								8.8 19.0 1.1 Liver normal.
10	0.14				0.10				0.10			5.0 27.0 2.7 Liver very pale.
20	0.14								0.10			4.6 21.0 2.2 Liver normal.
17	0.13								0.11			3.8 18.0 2.3 Liver normal.

\* Experiment of 4 hours.

\* Experiment of 4 hours.

† Experiment of 3 hours.

Referring to the data in Table 2, the results of experiments with hydrazinized rabbits, it is at once apparent that no conclusive statement may be made since with these animals the figures obtained show an utter lack of uniformity.<sup>8</sup> At first it was thought that there existed a relation between the blood sugar content and the degree of sugar disappearance from the perfusion fluid, that is, that at a definite point in the decline of blood sugar content sugar transformation might be much more rapid than at any previous or subsequent period. The results as shown in Table 2 lend no support to such an hypothesis.

On the other hand, the data from experiments 10, 20 and 17, where in spite of hydrazine administration blood sugar content remained unchanged, furnish a possible clue to the significance of the distinctly augmented sugar disappearance from the perfusing fluid. In the experiments enumerated above the figures cited are much larger than those for the controls given in Table 1, and one may ask whether these larger figures obtained may not stand in direct relation to the nutritive condition of the animal, that is, with respect to its glycogen content or carbohydrate. To test this hypothesis experiments 33, 31, 34, 35, 32, 38, 36, and 39, Table 1, were carried through, and it is at once evident that simple starvation, without the intervention of hydrazine intoxication, is sufficient to account for the high figures now and then obtained in hydrazinized animals. If the lack of glycogen, one of the most obvious changes accompanying starvation, is alone responsible for the augmented sugar disappearance figures obtained one is at a loss to understand why the animals showing typical hydrazine symptoms, *i.e.*, low blood sugar, should exhibit figures for sugar disappearance characteristic of animals deviating little from the normal. Again, if the amount of glycogen is the sole determining factor it is difficult to understand why a period of three or four days' starvation, a period hardly sufficient to deplete entirely the glycogen store, should lead to results in sugar disappearance as great or greater than those obtained after a period of eight days' starvation.

Pursuing the idea of glycogen store in the tissues as a significant factor, experiments with well-fed animals were made, see Table 1,

<sup>8</sup> Cf. results obtained by Patterson and Starling with the dog's heart, *Journ. of Physiol.*, xlvii, p. 137, 1913.

experiments 24, 26, 40, 41, 42, 43, and 37. It is evident from these results that the hearts of well-fed rabbits in general yield results significantly lower than those obtained with starved animals. It is also apparent that at times a rabbit in good nutritive condition will furnish a high figure, see experiment 42, Table 1.

Another possible explanation for the behavior discussed above is the influence of the degree of contractility of the heart muscle. To eliminate so far as possible contractility of the heart muscle the hearts were perfused with calcium-free Locke's solution, see experiments 45, 48, 46, and 47. The figures obtained are indeed conflicting and afford little or no evidence in support of the idea that glycogen is the determining factor for increased sugar disappearance from the perfusion fluid. On the other hand, the data indicate definitely that the sugar disappearance is little different in the strongly beating heart than in the non-beating organ relaxed to its maximum, thus corroborating the conclusion of Locke.

#### CONCLUSIONS.

Hydrazine subcutaneously administered to rabbits causes no greater quantity of sugar to disappear from a solution perfused through the heart than that obtained with hearts of non-hydrazinized animals in a comparable nutritive condition.

With non-hydrazinized rabbits the nutritive condition, and hence possibly the quantity of glycogen present in the tissues, as indicated by starvation experiments, is apparently a factor in determining the sugar disappearance mentioned.

In confirmation of the work of previous investigators it is shown that sugar disappearance from the perfusion fluid with the beating heart is little different from that obtained when the heart is not beating and is relaxed to a maximum.

These experiments therefore fail to answer the question as to the cause of diminution of blood sugar content after hydrazine administration.

## ON THE SEVERAL FACTORS OF ACID EXCRETION.

BY LAWRENCE J. HENDERSON AND WALTER W. PALMER<sup>1</sup>

(From the Wards and the Chemical Laboratory of the Massachusetts General Hospital, Boston.)

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The present paper is a continuation of the program for the study of the excretion of acid which, two years ago, was set forth by one of us.<sup>2</sup> We have previously investigated and reported upon the hydrogen ion concentration of urine, its normal and pathological range, variation and average value,<sup>3</sup> the influence of ingested acids and alkalies thereon,<sup>4</sup> as well as certain clinical developments of these studies.<sup>5</sup> We have now extended our observations to the magnitude of the excretion of acid and its two moities, as previously defined: First, the excess of acid radicals above the amount originally associated in the blood with the urinary bases; and secondly the urinary ammonia. These observations have been made in connection with further observations upon the hydrogen ion concentration and the urinary volume, with frequent observations upon the diet, and in all cases they deal with the full daily (24-hour) quantity of urine. Here and

<sup>1</sup> Henry P. Waleott Fellow in Clinical Medicine, Harvard Medical School.

<sup>2</sup> L. J. Henderson: A Critical Study of the Process of Acid Excretion, this *Journal*, ix, p. 403, 1911.

<sup>3</sup> L. J. Henderson and W. W. Palmer: On the Intensity of Urinary Acidity in Normal and Pathological Conditions, this *Journal*, xiii, p. 393, 1913.

<sup>4</sup> L. J. Henderson and W. W. Palmer: On the Extremes of Variation of the Concentration of Ionized Hydrogen in Human Urine, this *Journal*, xiv, p. 81, 1913.

<sup>5</sup> W. W. Palmer and L. J. Henderson: Clinical Studies on Acid Base Equilibrium and the Nature of Acidosis, *Arch. of Int. Med.*, xii, p. 153, 1913; L. H. Newburgh, W. W. Palmer and L. J. Henderson: A Study of Hydrogen Ion Concentration of the Urine in Heart Disease, *ibid.*, xii, p. 146, 1913; L. H. Newburgh: The Treatment of Cardiac Edema with Alkali and Salt, *Boston Med. and Surg. Journ.*, clxix, p. 40, 1913.

now we are concerned only with individuals supposed to be normal with respect to the acid-base regulatory function: young physicians and adult patients in the hospital in whom no renal, circulatory, or general metabolic disturbance was known to exist. In all, one hundred and twenty-two separate urinary samples from sixteen individuals are here reported.

#### OBSERVATIONS.

The estimations of the hydrogen ion concentration expressed as the negative logarithm thereof,  $H^+$ , and of titratable acid excretion, A, have been carried out in the manner previously described.<sup>6</sup> The process of titration is a simple one. Ten cc. of a 0.1 molal solution of monosodium phosphate, one part, and disodium phosphate, five parts (yielding a hydrogen ion concentration of approximately 7.4) are introduced into a 250-cc. flask, diluted to 250 cc. with distilled water and 0.2 cc. of a 2 per cent aqueous solution of neutral red is added. Ten cc. of urine are then similarly diluted, neutral red is added and  $\frac{N}{10}$  sodium hydrate is run in until the color matches that of the phosphate solution. The use of potassium oxalate has been found unnecessary.

Ammonia has been determined according to Folin's method.<sup>7</sup>

All observations have been made in duplicate, invariably with close agreement in the results. The observations follow (table I).

These data may be arranged in four groups of twenty, forty-four, twenty-nine and twenty-nine specimens respectively corresponding to the intensity of the acidity. The average values of the several characteristics in each group, together with their average values for all the 122 instances are brought together in table II (p. 310).

Finally in table III (p. 311) we have assembled the average values of the different characteristics of each group expressed as per cents of the average for the whole 122 cases.

<sup>6</sup> This *Journal*, ix, p. 403, 1911; *ibid.*, xiii, p. 393, 1913.

<sup>7</sup> O. Folin and A. B. Macallum: The Determination of Ammonia in Urine, this *Journal*, xi, p. 523, 1912.

TABLE I.

CASE NO.	AMOUNT CC.	$\frac{+}{H}$	ACID CC. $\frac{N}{10}$	$NH_3$ CC. $\frac{N}{10}$	TOTAL ACID CC. $\frac{N}{10}$	A $NH_3$
1	1050	5.7	400	375	775	1.07
	1240	5.7	403	445	848	0.90
	1300	5.4	470	517	987	0.91
	1200	5.7	450	475	925	0.95
	1340	6.0	325	405	730	0.80
	1200	6.0	290	480	770	0.60
	1200	6.7	204	290	494	0.70
	1520	6.0	360	515	875	0.70
	1220	5.7	270	390	660	0.69
	1000	5.7	340	430	770	0.79
	1000	5.7	347	455	802	0.76
	1000	5.8	362	450	812	0.80
2	900	6.5	202	190	392	1.06
	760	6.5	163	179	342	0.91
	1120	5.7	410	358	768	1.14
	960	6.7	195	218	413	0.90
	920	6.0	184	230	414	0.80
	1100	5.7	340	320	660	1.06
	740	5.4	280	314	594	0.89
3	1000	6.0	222	360	582	0.62
	820	5.5	410	600	1010	0.68
	1100	6.3	195	390	585	0.50
	1500	5.7	315	535	850	0.59
	1140	6.0	150	290	440	0.52
4	1000	5.6	385	285	670	1.35
	1200	5.4	440	378	818	1.16
	1340	6.3	450	430	880	1.05
	2100	6.3	330	465	795	0.71
	1600	6.1	290	450	740	0.65
	2000	6.0	320	420	740	0.76
	1600	6.3	237	330	567	0.72
	1500	6.1	285	385	670	0.74

TABLE I—Continued.

CASE NO.	AMOUNT CC.	+ H	ACID CC. $\frac{N}{10}$	NH <sub>3</sub> CC. $\frac{N}{10}$	TOTAL ACID CC. $\frac{N}{10}$	A NH <sub>3</sub>
5	620	6.5	155	210	365	0.74
	800	7.0	115	270	385	0.43
	1260	5.8	505	560	1065	0.90
	620	6.7	100	230	330	0.43
	1080	5.7	290	500	790	0.58
	840	5.6	234	290	524	0.81
	700	5.7	206	256	462	0.80
	1175	5.8	390	400	790	0.98
6	1460	6.0	460	500	960	0.92
	1640	6.0	450	515	965	0.88
	1000	5.6	400	400	800	1.00
	1040	5.7	330	380	710	0.87
	1350	6.0	304	319	623	0.95
	1040	6.0	242	185	427	1.30
	1400	6.1	280	385	665	0.73
	1275	5.8	276	264	540	1.05
	1040	5.5	292	258	550	1.13
	1140	6.1	222	220	442	1.01
	1200	6.0	260	308	568	0.85
7	1150	5.5	470	405	875	1.15
	1000	5.7	348	780	1128	0.45
	960	6.0	214	550	764	0.40
	1440	6.1	236	550	786	0.43
	1000	6.5	155	390	545	0.40
	1480	6.5	191	450	641	0.42
	850	5.8	155	330	485	0.47
	700	7.0	42	150	192	0.28
8	575	5.3	234	390	624	0.60
	820	6.0	274	292	566	0.94
	600	5.8	87	152	239	0.57
	850	6.0	80	275	355	0.29
	600	6.1	100	136	236	0.74
	600	5.8	105	200	305	0.53
	620	5.6	154	220	374	0.70
	800	5.7	233	270	503	0.86

TABLE I—Continued.

CASE NO.	AMOUNT CC.	$\frac{+}{H}$	ACID $\frac{N}{10}$ CC. $\frac{N}{10}$	$NH_3$ $\frac{N}{10}$ CC. $\frac{N}{10}$	TOTAL ACID $\frac{N}{10}$ CC. $\frac{N}{10}$	$\frac{A}{NH_3}$
9	1020	6.0	200	540	740	0.37
	1300	5.7	360	590	950	0.61
	820	6.3	190	470	660	0.40
	975	5.7	210	420	630	0.50
	1000	5.8	161	375	536	0.70
	1400	6.1	182	430	612	0.42
10	2500	6.7	188	330	518	0.57
	2100	6.3	328	370	698	0.89
	1720	5.7	394	475	869	0.83
	1800	5.8	332	535	867	0.62
	1520	6.3	225	325	550	0.69
	2000	6.0	280	386	666	0.73
	1220	5.8	292	330	622	0.89
	1900	6.5	222	325	547	0.68
	2700	5.8	334	480	814	0.68
	1700	5.8	315	376	691	0.84
11	850	5.7	350	280	630	1.25
	900	6.0	180	200	380	0.90
	1000	6.5	245	220	465	1.11
	1145	5.6	380	320	700	1.19
	1000	5.5	362	330	692	1.10
12	1350	5.4	216	430	646	0.51
	1500	5.7	274	400	674	0.69
	1700	5.8	340	450	790	0.76
	1560	6.0	290	400	690	0.73
	2280	6.3	300	475	775	0.64
	1840	6.0	290	357	647	0.81
	1560	7.0	156	290	446	0.54
	1420	5.7	278	380	658	0.73
	1300	5.5	288	355	643	0.81
13	950	6.0	228	195	432	1.17
	1000	5.7	370	234	604	1.58
	1000	6.0	415	310	725	1.34
	1240	5.5	390	400	790	0.98
	1120	5.5	371	356	727	1.04
	1000	5.6	330	366	696	0.90

TABLE I—Continued.

CASE NO.	AMOUNT CC.	+ H	ACID $\frac{N}{10}$ CC.	$\text{NH}_3$ $\frac{N}{10}$ CC.	TOTAL ACID $\frac{N}{10}$ CC.	$\frac{A}{\text{NH}_3}$
14	1900	5.6	256	380	636	0.67
	1400	5.7	242	400	642	0.60
	1500	5.4	194	430	624	0.45
	1600	5.8	107	360	467	0.30
	1200	5.6	250	244	494	1.02
	1240	5.3	340	360	700	0.94
	1550	6.3	195	300	495	0.65
15	1350	6.7	244	540	784	0.45
	1850	6.7	242	430	672	0.52
	1560	6.3	333	520	853	0.64
	1900	6.5	238	565	803	0.42
	2100	7.0	336	550	886	0.61
	1400	7.0	316	440	756	0.72
16	875	5.5	300	310	610	0.97
	840	5.4	260	336	596	0.78
	820	5.1	315	350	665	0.90
	700	5.3	200	250	450	0.80
	900	5.1	306	314	620	0.97
	820	5.3	260	260	520	1.00

TABLE II.

NUMBER OF OBSERVATIONS	+ H	V	A	$\text{NH}_3$	A+ $\text{NH}_3$	$\frac{A}{\text{NH}_3}$
20	5.4	1026	320	367	687	0.87
44	5.7	1193	303	384	687	0.79
29	6.0	1259	263	365	628	0.72
29	6.6	1400	224	357	581	0.63
122	5.94	1231	278	370	649	0.75

TABLE III.

$\frac{+}{H}$	V	A	*NH <sub>3</sub>	A+NH <sub>3</sub>	$\frac{A}{NH_3}$
5.4	83.4	115.1	99.2	105.8	116.0
5.7	97.0	109.0	103.8	105.8	105.3
6.0	102.3	94.6	98.6	96.7	96.0
6.6	113.7	80.6	96.5	89.5	84.0

## DISCUSSION.

I. The observations on the intensity of acidity are in excellent agreement with those previously reported. The average value of the negative logarithm of the hydrogen ion concentration, 5.94, is in close accord with the average 6.03 of the 100 observations on normal urines which we have previously reported; the average of all 222, is 5.98. Hence it may be said with great certainty that under the general conditions of diet and activity with which we are here concerned the average intensity of acidity of normal urine amounts to  $6 \pm 0.1$ . From this mean individual normal cases depart considerably; thus the range for the daily quantity of urine is from about 5.1 to about 7, while samples of urine covering a shorter period may be as acid as 5 or as alkaline as 7.4.

II. Other things being equal, as was to be provisionally expected in our investigation, which approaches the statistical in the number of observations involved, the titratable acid excretion appears to be a function of the concentration of ionized hydrogen. In fact it has been previously shown that this quantity is due almost wholly to the presence of phosphoric acid in the urine.<sup>8</sup> Hence, in so far as the average concentrations of phosphoric acid in the four groups of urinary samples are constant, the values of A should be defined in their relation to  $\frac{+}{H}$  by the mass law. These considerations are in large part borne out by the facts. The average values of A do diminish steadily from 320 when the value of  $\frac{+}{H}$  is 5.4 to 224 when  $\frac{+}{H}$  has become 6.6. Moreover the variation corresponds in three of the four groups to what would be expected if A were due only to variation in the relative amount

<sup>8</sup> This *Journal*, ix, p. 403, 1911.

III. Unlike those of A the average values of  $\text{NH}_3$  are on the whole markedly constant. There appears to be a slight diminution<sup>+</sup> as  $\text{H}^+$  diminishes, but the variations are hardly outside the probable error, and cannot therefore be regarded for the present as real. This fact is not without significance. It proves conclusively that in the normal body nearly if not quite all the final regulation<sup>+</sup> of  $\text{H}^+$  through excretion falls upon the titratable excretion of acid, that is to say, upon the phosphates. Urinary ammonia is variable, to be sure, but in the normal individual it does not materially vary, for purposes of regulating the reaction of the blood, because it does not vary with the total acid excretion or with the urinary  $\text{H}^+$  but only otherwise in connection with variations in nitrogen metabolism, etc. This is one more confirmation of the view long ago put forward by one of us,<sup>9</sup> that the primary factors of regulation of the reaction of the body are the phosphates and carbonates.

IV. The values of the total excretion of acid,  $\text{A} + \text{NH}_3$ , call for no comment. They are made up of the values of the sum of a variable and a nearly constant quantity.

V. The values of the quantity  $\frac{\text{A}}{\text{NH}_3}$  have no especial significance not revealed by what has been above pointed out. We have, however, found this ratio a convenient one in our pathological studies, and here record it for that reason alone.

VI. Finally, an unexpected regularity in the volumes of the urine has been revealed.<sup>10</sup> On the average the volume increases steadily as  $\text{H}^+$  and A diminish. An inspection of the tables will show this variation to be apparently far too great for chance. This fact, though totally unexpected, is by no means wonderful, for it may be asserted as a necessary postulate of biology that no function of an organ is independent of any other, and the doctrine of probability would lead us to expect the dependence to be often great enough to be detected. But we have for the pres-

<sup>9</sup> L. J. Henderson: The Theory of Neutrality Regulation in the Animal Organism, *Amer. Journ. of Physiol.*, xxi, p. 427, 1908.

<sup>10</sup> It should be noted that case 8, where the daily volume averages low, was a rather undeveloped young man twenty-three years old.

ent no suggestion to make concerning the causes of this particular connection.

The facts concerning  $\overset{+}{H}$ , V, A and  $NH_3$  are for convenience expressed in graphic form, and this diagram may well serve as a summary of our results.

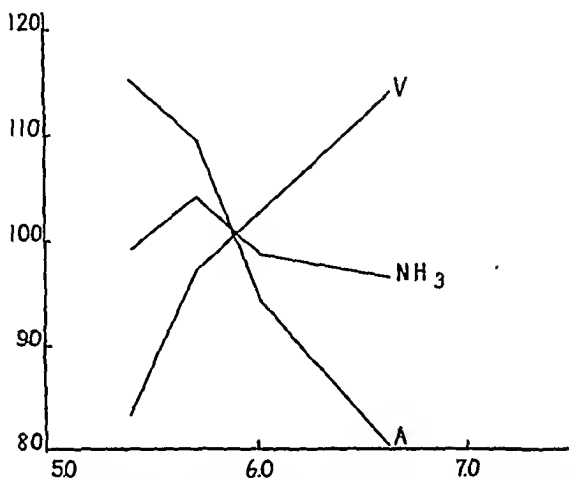


FIG. II. Ordinates, relative values of V, A and  $NH_3$ : Abscissas, reaction.



## THE STIMULATING INFLUENCE OF SERUM ON PANCREATIC AMYLASE.

By B. B. CROHN AND A. A. EPSTEIN.

(From the Pathological Laboratory, Mt. Sinai Hospital, New York City.)

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In attempting to investigate the influence of normal and diabetic serum on pancreatic amylase, the authors had occasion to observe that even small quantities of serum had a marked stimulating effect on the amylolytic activity of the extract of a dog's pancreas. This confirmed the findings of Pozerski<sup>1</sup> who had noted, this fact, and had undertaken to investigate the manner of this action of serum on the starch-splitting ferment. Wohlgemuth<sup>2</sup> had also been aware of this interesting observation of Pozerski's, and had made some experiments tending in the same direction.

The following series of tests was instituted in the effort to add some facts to those already known which bear upon the mechanism of this phenomenon.

*Technique.* An extract of the pancreas of a dog was made as follows: A medium sized dog was bled to death; pancreas dissected clear of any surrounding tissues, cut into small pieces and ground up in a mortar with washed sand; extracted in 100 cc. of 0.03 per cent ammonia for twenty-four hours; precipitated by the addition of 2 per cent acetic acid, and filtered through soft filter paper while standing on ice over night; the filtrate was neutralized with ammonia and diluted with distilled water to strength desired.

Starch solution was prepared by making a paste of "Kahlbaum Soluble Starch" in cold water, mixing it well, and adding to distilled water brought almost to boiling, but not allowed to boil.

<sup>1</sup> Pozerski: *Compt. rend. soc. biol.*, lv, p. 29, 1903.

<sup>2</sup> Wohlgemuth: *Biochem. Zeitschr.*, xxxiii, p. 303, 1911.

The serum used was separated from the dog's blood, or from human blood or human ascitic or pleural exudate. For routine use the serum was diluted with distilled water, as desired.

The general method of estimation of amylase was the slightly modified method of Wohlgemuth, as used by one of us in previous work.<sup>3</sup>

EXPERIMENT 1. Human blood serum: digestion, 24 hours.

A. Serum, diluted 1 : 4, 0.5 cc. in each tube. 2 per cent starch solution in amounts of 1, 2, 3, 5, 8 and 10 cc. *No digestion.*

B. Pancreatic extract, diluted 1 : 10, 0.5 cc. in each tube. 2 per cent starch solution in amounts of 0.5, 1.0, 1.5, 2, 4, and 6 cc. *Digestion = 1.75 cc.*

C. Serum, diluted 1 : 4, 0.5 cc. in each tube. Pancreatic extract, diluted 1 : 10, 0.5 cc. in each tube. 2 per cent starch solution in amounts of 2, 4, 6, 8, 10 and 12 cc. respectively. *Digestion complete in all tubes.*

This experiment demonstrates (1) the inactivity of the amylase of this particular specimen of serum; (2) an activity of pancreatic extract equivalent to 1.75 cc.; (3) an activity of the combined pancreatic extract and inactive serum equal to complete digestion of 12 cc., *i.e.*, at least seven times greater than that of pancreatic extract alone.

EXPERIMENT 2. A comparison of three sera (L1, K2, and K3), using in this case a fresh extract of dog's pancreas diluted 1 : 100 and 4 per cent starch solution. Digestion, 24 hours.

	L1	K2	K3
	cc.	cc.	cc.
A. Serum alone, diluted 1 : 4, 0.5 cc.....	0.5	0.75	0.5
B. Pancreatic extract alone, diluted 1 : 100.....	5.0	5.0	5.0
C. Combination of A and B.....	13.0	11.0	12.0

In this experiment we note (1) the comparative inactivity of serum amylase. (2) The constant value of pancreatic extract for amylase. (3) That the almost inactive serum combined with the pancreatic extract gives results which are much greater than the sum of the two acting separately.

<sup>3</sup> Crohn: *Amer. Journ. Med. Sci.*, cxiv, p. 393, 1912.

EXPERIMENT 3. Comparison of sera from pathological conditions in their ability to stimulate the amylolytic activity of pancreatic amylase.

	A SERUM ALONE DILUTED 1:4, 0.5 cc.	B PANCREATIC EXTRACT DILUTED 1:100, 0.5 cc.	COMBINA- TION OF A AND B
	cc.	cc.	cc.
1. Pneumonia.....	0.5	5	13
2. Diabetes mellitus (urine sugar-free).....	0.5	5	12
3. Chronic nephritis.....	0.75	5	11
4. Uremia.....	0.5	4	13
5. Pleural fluid, pleurisy with effusion.....	0.5	6	12
6. Pleural fluid (new growth of lung), non-hemorrhagic.....	0.5	6	13
7. Pleural fluid (new growth of lung), non-hemorrhagic.....	0.5	6	13

In this experiment the addition of 0.5 cc. of diluted serum more than doubled the activity of pancreatic extract. The origin of the serum did not seem to play a rôle for the activating power of all specimens appeared to be the same, barring slight fluctuation due to possible imperfection in technique.

EXPERIMENT 4. Activating effect of serum on human pancreatic secretion (duodenal contents, obtained by the Einhorn duodenal tube).

- A. Duodenal contents, normal case (dilution 1 : 10) 0.25 cc. used alone..... 3 cc.  
 The same + human serum diluted 1 : 4, 0.25 cc. used..... 20 cc.  
 B. Duodenal contents, cholelithiasis (dilution 1 : 10) 0.25 cc. used..... 6 cc.  
 The same + human serum diluted 1 : 4, 0.25 cc. used..... 30 cc.

This experiment shows marked activation of human pancreatic secretion by even a very small amount of human serum.

EXPERIMENT 5. Effect of varying the quantity of serum used, on its activating power. In each tube, 0.5 cc. of pancreatic extract, dilution 1 : 100, having a constant digestive value of 5 cc. of 2 per cent starch solution was used.

	CON- TROL	A	B	C	D	E	F
Amount of serum added.....	0	$\frac{1}{16}$ cc.	$\frac{1}{8}$ cc.	$\frac{1}{4}$ cc.	$\frac{1}{2}$ cc.	$\frac{3}{4}$ cc.	1 cc.
2 per cent starch solution digested.....	5	6	7	8.5	9	13	20

This experiment indicates that the activating power is proportionate to the quantity of serum used. Diluting the serum diminishes its activity in a like manner up to a certain limit of dilution.

EXPERIMENT 6. Effect of boiling the serum (sterilization) upon its activating power. Dilution of serum was 1 : 4; of pancreatic extract, 1 : 100. The results are expressed as cc. of 2 per cent starch solution digested in twenty-four hours.

1. 0.5 cc. serum, unboiled.....	1.5
2. 0.5 cc. serum, boiled 10 minutes.....	0
3. 0.5 cc. pancreatic extract.....	4.5
4. 0.5 cc. serum, unboiled, + 0.5 cc. pancreatic extract.....	15.0
5. 0.5 cc. serum, boiled 10 minutes + 0.5 cc. pancreatic extract	16.0

This experiment shows that boiling serum in no way diminishes its activating power. The slight difference after boiling may be due to increased concentration from evaporation.

EXPERIMENT 7. Effect of incubating serum and pancreatic extract, separately and together, on amylolytic activities. Dilution of serum was 1 : 4; of pancreatic extract, 1 : 100. Results are expressed as cc. of 4 per cent starch solution digested in 24 hours.

A. 0.5 cc. serum, unheated.....	0.5	
B. 0.5 cc. pancreatic extract, unheated.....	5	5
C. 0.5 cc. serum, incubated 24 hours at 38°C.....	0	
D. 0.5 cc. pancreatic extract incubated 24 hours at 38°C.....	0	
0.5 cc. pancreatic extract incubated 1 hour at 38°C.....	4	3
E. A + B.....	16	13
F. C + D, after incubation together 24 hours.....	11	11
C + D, after incubation together 1 hour.....		13.0
G. C + D, after incubation separately 24 hours.....	0	8
C + D, after incubation separately 1 hour.....		11.0

The following conclusions may be drawn from this experiment:

1. Pancreatic extract when incubated alone loses part of its amylolytic strength. 2. Serum when incubated alone loses part of its amylolytic strength. 3. When mixed together and incubated there is some loss of amylolytic strength. 4. When incubated separately and then mixed there is marked loss of amylolytic strength. 5. The presence of serum during incubation of pancreatic extract prevents in part the loss of amylolytic activity of the latter. 6. Serum after incubation is just as strong as

activating agent as before, although it loses its own natural amylolytic power.

EXPERIMENT 8. Effort to isolate the factor in the serum which activates the pancreatic extract.

A. Serum was boiled, precipitated with dilute acetic acid and filtered.

1. Whole serum, 1 : 4, + pancreatic extract, 1 : 100, digests 14 cc. 4 per cent starch solution in 24 hours.

2. Filtrate from boiled serum, 1 : 4, + pancreatic extract, 1 : 100, digests 12 cc. 4 per cent starch solution in 24 hours.

3. Protein precipitate from boiled, acidified serum has absolutely no activating power.

B. Dialysis experiments.

1. Undiluted pancreatic extract was dialyzed against normal salt solution for 24 hours. No amylase could be detected in the dialysate.

2. Human serum, diluted with equal volume of water was dialyzed against distilled water.

a. Pancreatic extract, 1 : 100, + distilled water digests 3 cc. of 2 per cent starch solution.

b. Pancreatic extract, 1 : 100, + 0.5 cc. of dialysate (24 hours) digests 5.5 cc. 2 per cent starch solution.

c. Pancreatic extract, 1 : 100, + 0.5 cc. of dialysate (48 hours) digests 7 cc. of 2 per cent starch solution.

d. Pancreatic extract, 1 : 100, + 0.5 cc. of serum (1 : 2) which had been dialyzed for 72 hours digests 11 cc. of 2 per cent starch solution.

e. Pancreatic extract, 1 : 100, + 0.5 cc. normal (not dialyzed) serum, 1 : 2, digests 20 cc. of 2 per cent starch solution.

3. Human serum was dialyzed against running water for 24 hours.

a. Control serum (not dialyzed) + pancreatic extract digests 11 cc. of starch solution.

b. Dialyzed serum + pancreatic extract digests 9 cc. of starch solution.

The results of experiment 8 show (1) that the activating substance in serum is not a coagulable protein but is present in the incoagulable fluid portion: (2) that amylase is not dialyzable: (3) that the activating substance is dialyzable.

These results directed our attention to the salts of serum which are the constituents which would probably be most easily removed from the serum by dialysis. They have been held by Wohlge-muth<sup>4</sup> to be responsible for the phenomenon as first noted by Pozerski. Experiment 9 was carried out to determine the activating power of the salts of serum.

<sup>4</sup> Wohlge-muth: *loc. cit.*

EXPERIMENT 9. Determination of activating power of salts of serum.

A. 1. Pancreatic extract + 0.5 cc. 0.9 per cent NaCl digests 6 cc. of 4 per cent starch solution.

2. Pancreatic extract + 0.5 cc. distilled water digests 6 cc. of 4 per cent starch solution.

B. 1. Pancreatic extract + 0.5 cc. Ringer's solution digests 5 cc. of 4 per cent starch solution.

2. Pancreatic extract + 0.5 cc. distilled water digests 3 cc. of 4 per cent starch solution.

C. Serum was dried, ashed and the residue taken up with water. The amount of this solution used in (1) corresponded to 0.5 cc. of serum.

1. Pancreatic extract + serum salts digests 7 cc. of 4 per cent starch solution.

2. Pancreatic extract + distilled water digests 3 cc. of 4 per cent starch solution.

From the results of this experiment we conclude that sodium chloride *per se* is not responsible for the activation: that a combination of chlorides of sodium, potassium and calcium as in Ringer's solution have activating power: and lastly that the salts derived directly from the serum account for a large part of the phenomenon.

EXPERIMENT 10. Determination of the activating power of other possible constituents of serum.

A. 1. Pancreatic extract + distilled water digests 3 cc. of 4 per cent starch solution.

2. Pancreatic extract + 0.1 cc. adrenalin, 1 : 1000, digests 3 cc. of 4 per cent starch solution.

3. Pancreatic extract + 0.5 cc. serum, 1 : 4, digests 7 cc. of 4 per cent starch solution.

4. Pancreatic extract + 0.5 cc. serum, 1 : 4, 0.1 cc. adrenalin, 1 : 1000, digests 8 cc. of 4 per cent starch solution.

B. 1. Pancreatic extract + distilled water digests 4 cc. of 4 per cent starch solution.

2. Pancreatic extract + 0.5 cc. 0.1 per cent lecithin solution digests 3 cc. of 4 per cent starch solution.

This experiment indicates that adrenalin may slightly increase the activating power of serum and that lecithin may exert a slight inhibiting action.

## GENERAL SUMMARY.

The results of these experiments may be summarized as follows: Small quantities of serum possess the power of increasing the amylolytic action of pancreatic extracts and pancreatic secretion by two, three or more than four-fold. This power is not diminished by boiling the serum nor by incubating it at 38°C. for many hours. The activating power of serum is lessened by dialysis. The salts of serum, in the proportion in which they exist in it, are largely responsible for the phenomenon: sodium chloride alone is not. Adrenalin may have a slight positive influence; lecithin has not. The origin of the serum plays no rôle.

If we attribute the greatest part of the activating power to the mineral salts, then the constancy of the phenomenon in different pathologic sera becomes intelligible, inasmuch as the mineral content of such sera varies but little. Roger<sup>5</sup> has found a similar ability, though to a lesser degree in sterilized saliva; Roger<sup>6</sup> also found the same for white of egg; Pozerski<sup>7</sup> for intestinal secretions. Probably in all the instances, the mineral content was the causative factor. Wohlgemuth<sup>8</sup> further has demonstrated that the serum after digestion is only slightly more activating in its effect than in the fasting person, also that the activating power of sera of different animals varies slightly in the following order: dog, sheep, rabbit, man, rat, horse. Nor is there any material difference between specimens of blood from different veins and different areas of the body. Wohlgemuth also ascertained that serum has the same influence on pancreatic amylase which it has on the amylase derived from other sources in the body, *e.g.*, organ extracts, saliva, etc.

Practically from the viewpoint of the physiologist, these facts are of great interest, as it has been found by one of us (Crohn)<sup>9</sup> that the pure duodenal contents have an amylolytic activity, which

<sup>5</sup> Roger: *Arch. de maladie de tube digestif et de la nutrition*, iii, p. 509, 1909.

<sup>6</sup> Roger: *Compt. rend. soc. biol.*, lxiv, p. 16, 1908.

<sup>7</sup> Pozerski: *Maly's Jahresberich'*, 1902, p. 400.

<sup>8</sup> Wohlgemuth: *loc. cit.*

<sup>9</sup> *Loc. cit.*

if estimated for the hydrolysis of the average carbohydrate intake of a normal man seems barely sufficient to digest it. But if we increase this coefficient two, three, or four times, by the presence of serum salts through the exudation of intestinal secretions, or the saliva, we can satisfactorily answer the query and explain why so very rarely starch appears in the stools, and why the pancreatic digestion so infrequently becomes insufficient for carbohydrates.

## AMINO-ACIDS IN NUTRITION AND GROWTH.<sup>1</sup>

BY THOMAS B. OSBORNE AND LAFAYETTE B. MENDEL.

WITH THE COÖPERATION OF EDNA L. FERRY AND ALFRED J. WAKEMAN.

(From the Laboratory of the Connecticut Agricultural Experiment Station  
and the Sheffield Laboratory of Physiological Chemistry in Yale  
University, New Haven, Connecticut.)

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The current trend of the investigation of the chemistry of nutrition is emphasizing the significance of the amino-acids as the fundamental factors in all problems in which hitherto the rôle of proteins has been involved. The remarkable success which has attended the efforts to supplant completely the proteins in the food intake by their ultimate products of hydrolysis—the so-called amino-acid “Bausteine”—has led to promising researches in which these food fragments have been followed beyond the alimentary barrier into the blood stream, to the tissues, and almost to their final destruction in the body. The question of protein synthesis has now become a problem of the biochemical department of amino-acids.

By Abderhalden's well-known experiments in this field of study it has been demonstrated that dogs (and even man) can be maintained in nitrogenous equilibrium, or, in some instances, can even gain weight and retain nitrogen when completely digested protein supplies the nitrogenous component of the diet.<sup>2</sup> Furthermore

<sup>1</sup>The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

<sup>2</sup>The earlier literature is referred to in Abderhalden's *Synthese der Zellbausteine in Pflanze und Tier*, Berlin, 1912; Abderhalden: *Fütterungsversuche mit vollständig abgebauten Nahrungsstoffen*, *Zeitschr. f. physiol. Chem.*, lxxvii, pp. 22-58, 1912; cf. also the experience of Henriques and Anderson: *Über parenterale Ernährung durch intravenöse Injektion*, *Zeitschr. f. physiol. Chem.*, lxxxviii, pp. 357-369, 1913, in which similar results were obtained by intravenous infusion of nutrients including proteins that were digested by trypsin and erepsin.

although without metabolism growth is impossible. If protein be administered beyond the requirement of the cells for repair and for growth, then the excess constitutes a "dynamic" quota which after deamination serves to furnish energy to the cells in the same fashion as do fat and sugar.<sup>5</sup>

In previous publications<sup>6</sup> we have presented clear evidence for the first time, we believe, that quite aside from the energy aspect and the quantitative features of the diet, certain proteins, notably the gliadin of wheat, may supply the nitrogenous needs of an animal in maintenance, yet be entirely inadequate for the purposes of growth. Still other proteins, as has long been known of gelatin and zein, fail to permit even the maintenance of animals in nutritive equilibrium. The remarkable fact that an animal can be maintained satisfactorily over very long periods, not merely a few days or weeks, on a diet which promptly becomes a promoter of growth by the mere substitution of another protein for gliadin, raises the question whether, after all, protein in the broader sense is necessarily destroyed by the so-called "wear and tear" of the body. The energy supply of the body can doubtless be obtained from any type of digestible food-stuff. Let us assume, for the purpose of argument, that a few definite amino-acids are indispensable in the functioning of certain cells or glands of the organism—that tryptophane, for example, is essential to the activities of some organ of internal secretion or in the elaboration of a necessary hormone, as already suggested by Willcock and Hopkins.<sup>7</sup> Tryptophane cannot be synthesized by the animal cells. It must therefore be furnished preformed to the individual, or obtained by decomposition of its tissue proteins, to permit the life activities to continue. Ordinarily this is accomplished by ingestion of tryptophane-yielding proteins. In accord with such a hypothesis it is

<sup>5</sup> Lusk: *The Elements of the Science of Nutrition*, 2d edition, 1909, p. 187.

<sup>6</sup> Osborne and Mendel: Beobachtungen über Wachstum bei Fütterungsversuchen mit isolierten Nahrungssubstanzen, *Zeitschr. f. physiol. Chem.*, lxxx, pp. 307-370, 1912; The Role of Gliadin in Nutrition, this *Journal*, xii, pp. 473-510, 1912; Maintenance Experiments with Isolated Proteins, *ibid.*, xiii, pp. 233-276, 1912; Wheeler, Ruth: Feeding Experiments with Mice, *Journ. of Exp. Zoölogy*, xv, pp. 209-223, 1913; Mendel: The Role of Proteins in Growth, *Trans. XV Internat. Cong. on Hygiene and Demography*, 1912.

<sup>7</sup> Willcock and Hopkins: Observations on the Effect of Adding Tryptophane to a Dietary in which Zein is the Sole Nitrogenous Constituent, *Journ. of Physiol.*, xxxv, pp. 88-103, 1906.

quite conceivable that what has hitherto been called the "wear and tear" (Abnutzungsquote) is in reality the demand for an amino-acid necessary for the construction of some non-protein nitrogenous substance essential for normal metabolism, rather than a requirement for the replacement of all the "Bausteine" which go to make up cellular proteins. On the basis of present evidence it is quite as logical to assume that the maintenance protein requirement is in reality a requirement for definite (as yet specifically undetermined) amino-acids that serve special physiological functions, as it is to insist that protein as such is demanded to repair a hypothetical destruction of the entire protein molecule. The latter may be regarded as ordinarily undergoing degradation only because by this method some essential amino-acid is liberated. Evidently zein and gelatin do not contain this; hence they cannot replace this non-protein nitrogenous wear and tear and consequently do not suffice for maintenance. Gliadin does contain the essential complex. The conspicuous qualitative distinction between the proteins referred to is the presence of the tryptophane group in gliadin and its absence from zein and gelatin. As we shall show, an animal can be maintained at constant weight for relatively long periods on a food containing gliadin as its sole protein factor, and at once be made to grow by adding lysine to such foods. Since synthesis of new protein is essential for the construction of new tissues, growth will be limited by any factor which prevents the synthesis of protein—in the above case, lysine. If such an interpretation of this failure to grow is correct, we should expect that these defective foods would fail to maintain the body-weight if protein is destroyed in the metabolism of maintenance unless the essential amino-acids can be synthesized as such. Inasmuch as animals on the above diets do not lose weight it is not improbable that very little, if any, protein is destroyed in such maintenance, and that the defective proteins furnished in these foods serve some other purpose than the reconstruction of protein (cf. Chart I, Rat 1113).

In respect to the nitrogenous requirements of the organism, growth sets a standard decidedly higher than that of maintenance; for certain amino-acids which cannot be synthesized by the growing organism must be furnished, not only for the maintenance functions but also for the construction of new tissue.

In respect to the precise character of the evidence which would be convincing in relation to protein synthesis from the amino-acids of the food, Abderhalden and Oppler wrote in 1907:

The problem of protein synthesis in the animal body from the simple "Bausteine" could be solved beyond criticism, if it were possible to cause young animals to increase in weight rapidly, and thereby produce an increase of their tissues, by feeding completely digested protein. Manifold obstacles interfere with such an experiment, and only an extensive experience will make it possible to furnish a convincing demonstration.<sup>8</sup>

Although Abderhalden and his collaborators have repeatedly referred to evidence of a new construction of tissues in some of their experiments in nutrition with amino-acids as the source of nitrogen, the actual gains reported are of a far different magnitude than those which are observed in normal growth such as, for example, we have repeatedly demonstrated in our studies on rats fed upon mixtures containing isolated proteins, and in those which form the subject of the present paper. This need not be interpreted as contradicting in any way the contention that growth, *i.e.*, a new synthesis of tissue protein, is possible from suitable intake of amino-acid mixtures; it merely means that more convincing data are still desirable. The best previous record which we recall was obtained by Abderhalden and Hirsch<sup>9</sup> who secured an increase equal in one case to 26 per cent of the original weight of the experimental animal, by feeding "crepton"—a digestion product prepared from meat. Abderhalden also states that in some of his experiments young dogs on diets of predigested foods have made considerable gains in weight—in one case 1000 grams, in another 1200 grams.<sup>10</sup> From this he argues that new formation of tissue has proceeded in considerable degree, and that the animal organism is able to construct all its cell components from simple "Bausteine." No temporary or transient gain of weight, and none which follows the depletion of the body by previous unsuitable nutritive conditions,

<sup>8</sup> Abderhalden and Oppler: *Zeitschr. f. physiol. Chem.*, li, p. 232, 1907.

<sup>9</sup> Abderhalden and Hirsch: Fütterungsversuche mit Gelatine, Ammonsalzen vollständig abgebautem Fleisch und einem aus allen bekannten Aminosäuren bestehenden Gemisch ausgeführt an jungen Hunden, *Zeitschr. f. physiol. Chem.*, lxxxi, pp. 323-328, 1912.

<sup>10</sup> Abderhalden: Fütterungsversuche mit vollständig abgebauten Nahrungsstoffen, *Zeitschr. f. physiol. Chem.*, lxxvii, pp. 22-58, 1912.

can be taken as evidence of true growth, for repair may be accomplished without necessarily implying actual synthetic processes in the sense intended. Real growth, consistently continued, manifests itself in characteristic increments of weight and size as exhibited in typical curves of growth.

It has been cleverly stated that "the tissue cells never know the food we eat." If the amino-acids are the true nitrogenous nutritive agents, and the ingested proteins appear to be equivalent in their nutritive efficacy for maintenance or growth to the amino-acids which they yield, the proteins themselves may be compared strictly from this viewpoint. In the light of present-day information the failures to replace protein by gelatin in this way have been due to inadequate knowledge in respect to the nature and proportions of the amino-acids which are linked together in the gelatin complex. Prior to the discovery of tryptophane the neglect to supply this derivative inevitably resulted disastrously; and even today, despite the recognition of the further lack of cystine and tyrosine, the composition of gelatin is not adequately known. The demonstration of the possibility of supplementing gelatin with amino-acids so as to render it complete in the sense of promoting growth remains to be accomplished. In so far as their chemical make-up is adequately known, the complete lack of, or partial deficiency of, individual proteins in any amino-acid can be supplemented by artificial additions to the imperfect protein, precisely as has been done in the case of inadequate mixtures of amino-acids. The addition of such amino-acids as are missing, or deficient in amount, ought, on this hypothesis, to make it possible to render adequate all proteins which are digestible in the alimentary tract. The history of earlier experiences in this field—of the attempts to supplement the then known deficiency of gelatin in particular by admixture of tyrosine, etc., in the hope of rendering it adequate for nutritive purposes, need not be detailed here.<sup>11</sup>

<sup>11</sup> See Escher: *Vierteljahresschrift der Naturforscher Gesellschaft in Zürich*, 1876, p. 36; Kaufmann, M.: *Arch. f. d. ges. Physiol.*, cix, p. 443, 1905; Rona and Müller: *Zeitschr. f. physiol. Chem.*, l, p. 263, 1906; Abderhalden: *ibid.*, lxxvii, p. 22, 1912.

## EXPERIMENTAL PART.

*Gliadin and lysine.*

That gliadin is adequate to supply the needs of the animal organism in respect to its nitrogenous requirements for maintenance over very long periods of time has been convincingly shown in our own experience.<sup>12</sup> Henriques<sup>13</sup> succeeded in maintaining the body-weight of rats unchanged for nearly a month on gliadin food, and claimed that if the protein is fed in abundance not only nitrogenous equilibrium, but even a retention of nitrogen in the body may result. We have found, however, that the method of collecting urine as devised by Henriques is attended by losses which leave the quantitative aspects of the subject uncertain. The failure to collect all of the nitrogen excreted would thus *appear* in favor of the storage of nitrogen. Growth was not reported by Henriques, and he failed to recognize the importance of the missing lysine.

The ready digestibility of gliadin is attested by numerous investigators.<sup>14</sup> Abderhalden was unable to replace proteins successfully by the products of the complete digestion of gliadin, even when the missing lysine was supplied in abundance.<sup>15</sup> He remarks that the proportion in which the various amino-acids are present is probably too little adapted for reconstruction of tissue.<sup>16</sup>

*We have succeeded in promoting growth at a normal rate when a maintenance ration containing gliadin as the sole protein was supplemented with lysine.* The success of the present series of feeding experiments has been greatly enhanced by the discovery that butter-fat promotes the growth of rats kept on the diets of isolated foodstuffs and "protein-free milk" which we have been accustomed to employ for several years.<sup>17</sup> Possible failures owing

<sup>12</sup> Cf. Osborne and Mendel: The Role of Gliadin in Nutrition, this *Journal*, xii, pp. 473-510, 1912.

<sup>13</sup> Henriques: Lässt sich durch Fütterung mit Zein oder Gliadin als einziger stickstoffhaltiger Substanz das Stickstoffgleichgewicht herstellen? *Zeitschr. f. physiol. Chem.*, lx, p. 105, 1909.

<sup>14</sup> Cf. Mendel and Fine: The Utilization of Proteins of Wheat, this *Journal*, x, p. 303, 1911.

<sup>15</sup> Cf. Abderhalden: *Synthese der Zellbausteine in Pflanze und Tier*, Berlin, 1912, p. 85.

<sup>16</sup> Abderhalden: *Zeitschr. f. physiol. Chem.*, lxxvii, p. 29, 1912.

<sup>17</sup> Osborne and Mendel: this *Journal*, xv, pp. 311-326, 1913; *ibid.*, xvi, pp. 423-437, 1913.

to the lack of suitable, as yet unknown accessory factors have been averted in this way.

The demonstration that the addition of lysine to the gliadin food serves to render this protein of wheat entirely adequate for the nitrogenous needs of growth is shown in Chart I, Rat 1113, in the appendix, in which the surprising effect of this amino-acid addition is in strong contrast with the mere maintenance effect of the diet without the lysine. The diets here referred to had the following composition:

*Gliadin food mixtures.*

(See Chart I.)

	WITHOUT LYSINE	WITH LYSINE <sup>18</sup>
	<i>grams</i>	<i>grams</i>
Gliadin <sup>19</sup> .....	18.0	17.46
Lysine.....	none	0.54
Protein-free milk.....	28.0	28.00
Starch.....	24.0	21.34
Butter-fat.....	18.0	18.00
Lard.....	12.0	14.00

How promptly the growing organism responds to the presence or lack of a nutritive unit indispensable for growth is even more strikingly shown in Chart I, Rats 1846, 1844, and 1850, in which the alternate addition and withdrawal of the lysine is followed by an immediate and unmistakable response in the character of the curve of body-weight.

We believe that these feeding trials, in conjunction with our demonstration of the almost complete cessation of growth on diets containing only lysine-free proteins, furnish the first and only conclusive demonstration that *lysine is indispensable for the func-*

<sup>18</sup> In preparing this food pure crystallized lysine dichloride was mixed with enough sodium carbonate to neutralize the hydrochloric acid combined with the lysine. This was then mixed with the finely ground gliadin, starch and protein-free milk. After thoroughly mixing all of these they were stirred into the melted lard and butter-fat. The final and complete mixing of all these ingredients with the fat was effected by passing the whole several times through a meat chopper.

<sup>19</sup> Regarding the content of lysine in our gliadin see Osborne and Mendel: The Role of Gliadin in Nutrition, this *Journal*, xii, pp. 473-510, 1912; also Osborne and Leavenworth: Do Gliadin and Zein Yield Lysine on Hydrolysis? *Ibid*, xiv, pp. 481-487, 1913.

tions of growth. They are supplemented by further evidence of the same sort in which the necessity for the same amino-acid is brought out in connection with the zein of maize, a protein likewise devoid of lysine (see Charts V and VI).

*Lysine in other proteins in relation to growth.*

The facts here established make it clear that, at least in so far as nutrition in growth is concerned, the normal construction of new tissues is limited by the factor of the supply of lysine. In the light of this, little is gained by emphasizing the quantitative aspects of the protein needs in growth as conspicuously as Rubner and others have done, unless the qualitative character of the protein available is kept clearly in mind. No amount of energy or protein, however abundant, has induced growth of our animals in the absence of lysine. The animal organism apparently cannot synthesize lysine, which is evidently not essential for maintenance in the sense of preservation of body-weight, though it is of course impossible to say that when this amino-acid is missing all functions are normally carried out. That the tissues either form a typical protoplasmic product, or none at all, now seems to be axiomatic in physiology. We may therefore reasonably assume that the growth of rats on our gliadin + lysine food represents the construction of typical tissue substance. It is obvious, furthermore, that the possibility of growth must be limited, among other things, by the amount of lysine available. How widely proteins differ in their content thereof, is shown by the following data taken from the best analyses at hand:

*Lysine in proteins.<sup>20</sup>*

	per cent		per cent
Laetalbumin.....	8.10	Glutelin, maize.....	2.93
Halibut muscle.....	7.45	Glutenin, wheat.....	1.92
Ox muscle.....	7.59	Edestin, hemp-seed.....	1.65
Casein, cow's milk.....	7.61	Amandin, almond.....	0.72
Vitellin, egg-yolk.....	4.81	Gliadin, wheat.....	0.16
Crystallized albumin, hen's egg	3.76	Hordein, barley.....	0.00
Legumin, pea.....	4.98	Zein, maize.....	0.00
Phaseolin, kidney bean.....	4.53		

<sup>20</sup> These data have been obtained by Kossel and Kutscher's method. The recent investigations of Van Slyke and his associates indicate that these figures are probably a little low. Cf. this *Journal*, xvi, p. 531, 1914.

It is a teleologically interesting fact brought out by the foregoing figures that those proteins, like casein, lactalbumin, and egg vitellin, which are in nature concerned with the growth of animals, all show a relatively high content of lysine. Further presumptive evidence in respect to the rôle of lysine here portrayed is furnished by experiments in which the relative efficiency of different proteins in transforming a diet that is inadequate for growth into one that promotes growth is clearly brought out. Zein, like gliadin, is devoid of lysine-yielding complexes and cannot promote growth. It can be made to suit the needs of growth better, as we shall see later, if all of its amino-acid deficiencies are suitably made good. When other proteins are furnished in addition to zein in the diet, the proportions required to render the ration satisfactory for growth are unlike with respect to the quantities necessary to permit the animal to make normal growth with a minimum of the supplementary protein. In other words, the zein diet may be rendered efficient for normal growth by replacing a part of the zein with other proteins containing the amino-acids which zein lacks. The minimal proportion thus required is not the same for each protein, but is determined by the proportion of that amino-acid, thus supplied, which is present in the least amount in the added protein. Lactalbumin and edestin, for example, are in strong contrast in respect to their content of lysine (see table p. 334). Either of these proteins, like certain others that we have tried, will supplement a zein ration so as to permit adequate growth. The proportion necessary for this purpose is, however, different for the two proteins in accord with their unlike lysine yield. Thus when the lysine-rich lactalbumin is used, a 25 per cent replacement suffices for perfect growth, whereas in the case of edestin, low in lysine, a similar substitution results in no increment of weight. In order to obtain growth approximating the normal it is necessary to replace one-quarter of the zein of our standard ration with lactalbumin, or three-quarters with edestin (Chart II). The quantitative insufficiency of smaller proportions of edestin is shown in Chart III. That the lysine is the controlling factor is demonstrated by Rats 1807, 1799 and 1531 in Chart III which also show the effect of addition of lysine to an inadequate proportion of edestin. Such experi-

ments have been duplicated repeatedly, and speak plainly for the dominating importance of lysine in the synthetic nutrition of growth.

### *Zein and amino-acids.*

Zein presents even more striking differences than gliadin in its amino-acid make-up when compared with the other proteins commonly present in foods. The greatest interest has centered around the entire absence of glycocoll, tryptophane and lysine; for feeding experiments with zein were expected to shed light on the important question of amino-acid synthesis by the animal. The deviations of zein from other proteins which we have used extensively for feeding are further emphasized below:

*Comparative composition of proteins.*

AMINO-ACIDS	ZEIN (MAIZE)	GLIADIN (WHEAT)	CASEIN (MILK)	LACTAL- BUMIN (MILK)	EDESTIN (HEMP- SEED)
	per cent	per cent	per cent	per cent	per cent
Glycocoll.....	0.00	0.00	0.00	0.00	3.80
Alanine.....	13.39	2.00	1.50	2.50	3.60
Valine.....	1.88	3.34	7.20	0.90	6.20
Leucine.....	19.55	6.62	9.35	19.40	14.50
Proline.....	9.04	13.22	6.70	4.00	4.10
Phenylalanine.....	6.55	2.35	3.20	2.40	3.00
Aspartic acid.....	1.71	0.58	1.39	1.00	4.50
Glutaminic acid.....	26.17	43.66	15.55	10.10	18.74
Serine.....	1.02	0.13	0.50	?	0.33
Tyrosine.....	3.55	1.50	4.50	2.20	2.13
Cystine.....	?	0.45	?	?	1.00
Histidine.....	0.82	1.49	2.50	1.53	2.19
Arginine.....	1.55	3.16	3.81	3.01	14.17
Lysine.....	0.00	?	7.61	8.10	1.65
Tryptophane, about.....	0.00	1.00	1.50	+	+
Ammonia.....	3.64	5.22	1.61	1.32	2.28
	88.87	84.72	66.92	56.46	82.28

The failure of animals to grow or even be maintained when zein is the sole form of nitrogenous food-intake for any considerable period has been widely recognized. Feeding experiments conducted with rats by Henriques<sup>21</sup> show in some protocols surprising-

<sup>21</sup> Henriques: *Zeitschr. f. physiol. Chem.*, lx, p. 108, 1909.

ly small decline in body-weight during the relatively short periods of two weeks, or less, within which they were continued. This investigator contents himself with the conclusion that nitrogenous equilibrium cannot be obtained with zein as the sole source of nitrogen in the diet. The digestibility of zein has frequently been found to be quite poor. This seems, however, to be in good measure due to the physical character of the isolated zein which tends to assume a hard, resistant form, even when finely ground. "Coefficients of digestibility" ranging from 50 to 90 per cent are reported for corn proteins, but there are few data derived from experiments conducted under ideal conditions.<sup>22</sup> In our own work we have long taken the precaution to hydrate the zein by incorporating a little water in the food; and we cannot charge the poor nutritive results to undue lack of absorption of the digestion derivatives of zein.

The inability of zein food to maintain animals adequately is attested by the experience of Willcock and Hopkins.<sup>23</sup> In experiments with mice they found that zein "has no power whatever of maintaining growth in the young animal; loss of weight begins the moment it forms the sole nitrogenous supply." They further remark:

The addition of the missing tryptophane group has, it is also clear, no power to convert such loss into equilibrium or gain: a fact possibly due to other deficiencies in the zein molecule, such as the absence of lysine, or the lack of some other amino-acid not yet observed. There was no close relationship in our experiments between the loss of weight and the length of survival period. In many individual cases the mice upon tryptophane lost a considerably larger percentage of their weight before death than, on the average, did those without it. Such differences may be largely due to differences in the nutritional condition of individuals at the outset, but the results appear to show that death was not determined by a critical percentage loss. On the other hand the figures show that, on the average, the loss of body-weight was slower with tryptophane than without it. But this result might well be expected, even if the tryptophane administered undergoes utilization without directly contributing to tissue formation or structural maintenance. If it serves as a basis for the elaboration of a substance absolutely necessary for life—something, for instance, of an importance equal to that of adrenaline—then, in starvation, or when it is

<sup>22</sup> Cf. Mendel and Fine: this *Journal*, x, p. 345, 1911.

<sup>23</sup> Willcock and Hopkins: *Journ. of Physiol.*, xxxv, pp. 88-103, 1906.

absent from the diet, a supply is likely to be maintained from the tissue-proteids; the demand for it would become one of the factors determining tissue breakdown. In the case of young animals which directly benefit from the addition of a protein constituent, otherwise absent from their diet, to the extent of a well nigh doubled life, and marked improvement in general condition, but at the same time steadily lose, instead of gaining, weight, the utilization of the constituent would seem to be of some direct and specific nature.

McCollum<sup>24</sup> appears to have had somewhat better success in feeding pigs with zein-food. He maintained an animal unchanged in body-weight for three weeks on zein as the sole protein (see McCollum, Table III, p. 225) and remarks: "All previous efforts to induce growth in animals by feeding zein as the sole source of protein have failed entirely, but the pig appears to be exceptionally efficient in the utilization of foodstuffs, so it seemed possible that more favorable results might be met with in the case of zein."

We have now accumulated the results of a large experience in feeding zein to both adult and growing rats with the uniform consequence of decline, when this protein forms the sole nitrogenous component of the dietary. Reference to Chart IV will suffice to show the general character of numerous trials during intervals of several years with outcomes differing only in the greater or lesser rapidity of the decline. We may point out that our zein has always been purified with care, so that contamination with other corn proteins has been avoided. The importance of this may be realized in view of the fact that small additions of these are sufficient to prevent decline. Furthermore we have not attempted to induce our animals at the same time to consume undue amounts of non-nitrogenous foods, as can so effectively be done in the case of the pig, with the result of preventing fall in body-weight for surprisingly long periods even in the absence of any protein intake.<sup>25</sup>

The best evidence of the unique rôle of *tryptophane* and its indispensability to nutrition in preserving maintenance in the organism, is shown by comparing the curves of body-weight of rats furnished food containing zein + tryptophane with those of rats on foods containing zein without this addition. (See Charts IV

<sup>24</sup> McCollum, E. V.: *Amer. Jour. of Physiol.*, xxix, pp. 215-236, 1911.

<sup>25</sup> Cf. McCollum, E. V.: *Amer. Journ. of Physiol.*, xxix, pp. 215-237; Grafe, E.: *Zeitschr. f. physiol. Chem.*, lxxxviii, pp. 389-424, 1913.

and V.) It is to be noted that those rats which had tryptophane declined slowly except 1892 and 1895 which were almost perfectly maintained for more than ninety-four and sixty-six days respectively. These two rats were supplied with butter-fat in place of a part of the lard in their diet. The beneficial effects of this addition were not known at the time the other experiments were in progress. That this factor had no favorable influence when zein alone was fed is shown in Chart IV by Rats 684, 1773, 1890, and 710 which declined as rapidly as the others, although their food contained 18 per cent of butter-fat.

In the comparable experiments with mice which Miss Wheeler<sup>26</sup> conducted at our suggestion, the beneficial effect of tryptophane additions to zein food was more pronounced than appears in the records of Willcock and Hopkins. Mice fed by the latter on zein died before the twelfth day; those fed with zein + tryptophane were alive and active on the sixteenth day when the experiment was terminated, but they had lost weight. In Miss Wheeler's experiments mice fed with zein lost on the average one-third of their weight in twenty-five days; while two mice which had an addition of tryptophane equal to 3 per cent of the zein fed, lost only about one-fifth of their original weight by the fiftieth day. It is of interest to note that in Miss Wheeler's experiments gelatin was far less effective in its nutritive powers than was zein; and that with a diet containing equal parts of zein and casein satisfactory maintenance of mice was secured.

The lack of tryptophane can also be made good, as might be expected, by supplementing the zein ration with proteins which do contain this lacking amino-acid. Thus even gliadin stopped the decline (see Chart I, Rat 1113). The relative efficiency of different proteins in preventing the failure with zein apparently depends to a dominant degree, in so far as maintenance is concerned, on their comparative yield of tryptophane.

Where growth is involved in addition to maintenance, the lysine factor, as well as others, not yet more accurately ascertained, must also be taken into account. Here, then, is evidence of the relative economy of different proteins in maintenance, based upon the content of one or more of the amino-acids essential for the proper func-

<sup>26</sup> Wheeler, Ruth: Feeding Experiments with Mice, *Journ. of Exp. Zoölogy*, xv, pp. 209-223, 1913.

tioning of the organism (in so-called maintenance) or for new tissue construction (in growth). *Obviously the relative values of the different proteins in nutrition are based upon their content of those special amino-acids which cannot be synthesized in the animal body and which are indispensable for certain distinct, as yet not clearly defined processes which we express as maintenance or repair.*

This is a viewpoint somewhat different from the general hypothesis of Abderhalden, that the greater the similarity of the molecule of food protein to that of the body proteins, the greater will be the food value to the animal. It likewise differs from the conclusions drawn by Michaud<sup>27</sup> in his attempt to substantiate the foregoing. He states that the protein minimum is most readily attained if the protein of the same species (*körpereigenes Eiweiss*) is fed, and that more is required whenever protein of a different amino-acid make-up is fed. The implication, of course, is that the protein minimum represents a total destruction of protein which can best be made good by supplying all of the lost amino-acids in precisely the proportions in which they existed in the catabolized body protein. Bearing in mind the possibility, developed in our introductory remarks, that the destruction in maintenance (apart from any need of energy not supplied by non-protein sources) may represent chiefly a device for getting one or more amino-acids essential for some regulatory, or similar function, rather than *all* that are needed for reconstruction of tissue destroyed, Michaud's findings can be explained quite as well upon this hypothesis. We need merely point out that dog-meat, casein, edestin, "glidin" and serum proteins probably differ greatly in their yield of the indispensable amino-acids. When therefore an animal is put upon short protein rations it may well be that those richest in tryptophane, for example, will best fill the minimum maintenance requirement. Muscle tissue which is richer than edestin in lysine, as happened in Michaud's experiments, ought to prove more economical. It is not improbable that the relatively successful records of dog's meat in Michaud's experiments can be equalled with other proteins, like lactalbumin, which can scarcely be called "*körpereigenes Eiweiss*." The problem of protein minimum needs to be approached from new standpoints.

<sup>27</sup> Michaud: *Zeitschr. f. physiol. Chem.*, lix, p. 405, 1909.

With the indispensability of tryptophane for maintenance, and of lysine for growth, thus emphasized, we may expect that the addition of *both* of these amino-acids to zein food will result in growth. This expectation has been fulfilled by the experiments planned (see Chart VI). The respective parts played by the two amino-acids is here clearly brought out. *These are, we believe, the first successful attempts to grow animals on a diet in which zein forms the sole protein.* If we compare Charts VII, VIII and III which show the relative effect of replacing one-fourth of the zein with lactalbumin, casein, and edestin respectively it will be seen that this small addition of lactalbumin has furnished all the factors required for normal growth. This protein mixture therefore undoubtedly contains sufficient tryptophane and lysine to satisfy the normal requirements of the growing animal. Since casein yields nearly as much lysine as does lactalbumin, we assumed that the failure of an equal addition of casein was due to a relative deficiency in tryptophane. That this assumption was correct is shown by Rats 1808 and 1809 in Chart VIII; the addition of a small amount of tryptophane to the previously inefficient zein + casein diet at once resulted in rapid growth. Similarly we assumed that the failure to grow on the comparable zein + edestin food was caused by the relatively small amount of lysine yielded by edestin. Here, too, the correctness of our assumption is demonstrated by Rats 1807 and 1799 (Chart III) in which the addition of lysine rendered the food mixture adequate for growth at a normal rate. These experiments are further of chemical interest in indicating, as the result of this biological test, that edestin is richer in tryptophane than casein—a fact hitherto unappreciated.

The growth of rats on a food of zein + lysine + tryptophane has not always been as rapid and prolonged as we might expect. We are by no means prepared to maintain that the final solution of the proportion of amino-acids requisite for the growth of rats has been determined. Newer trials may indicate the desirability of increasing the proportion of arginine present in zein foods; and still other adjustments may be required to promote ideal growth in this or different species. The way to successful investigation has been opened.

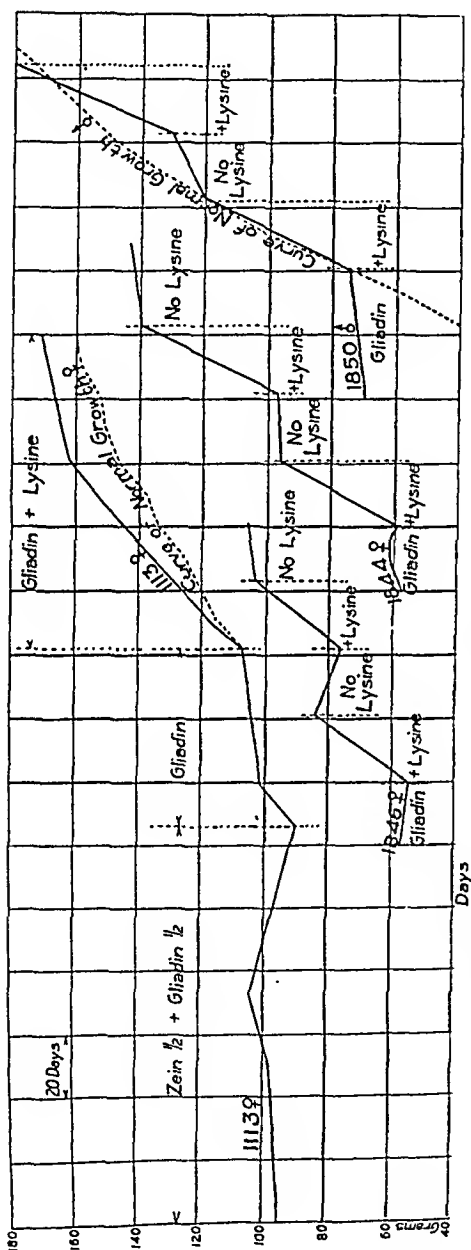


CHART I. INDISPENSABILITY OF LYSINE FOR GROWTH. This chart shows the failure to grow on diets containing gliadin as the sole protein; the immediate resumption of growth when lysine equivalent to 8 per cent of the protein is added to the food; and the equally prompt cessation of growth when the addition of lysine is stopped.

Animals cannot be maintained on zein food alone. Inasmuch as gliadin does not yield more than insignificant amounts of lysine, practically no growth is made on foods containing gliadin as the sole protein. A food containing equal parts of zein and gliadin serves to maintain body-weight. See Rat 1113. This indicates that not all of the amino-acids yielded by proteins are necessary for maintenance; otherwise we would expect destruction of tissue and fall of body-weight in the experiment just cited.

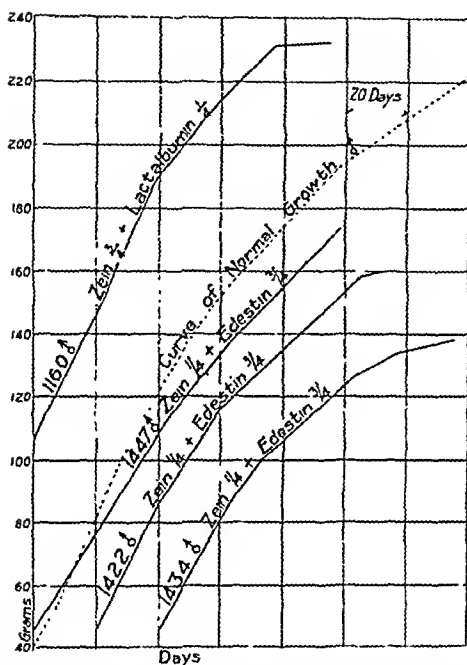
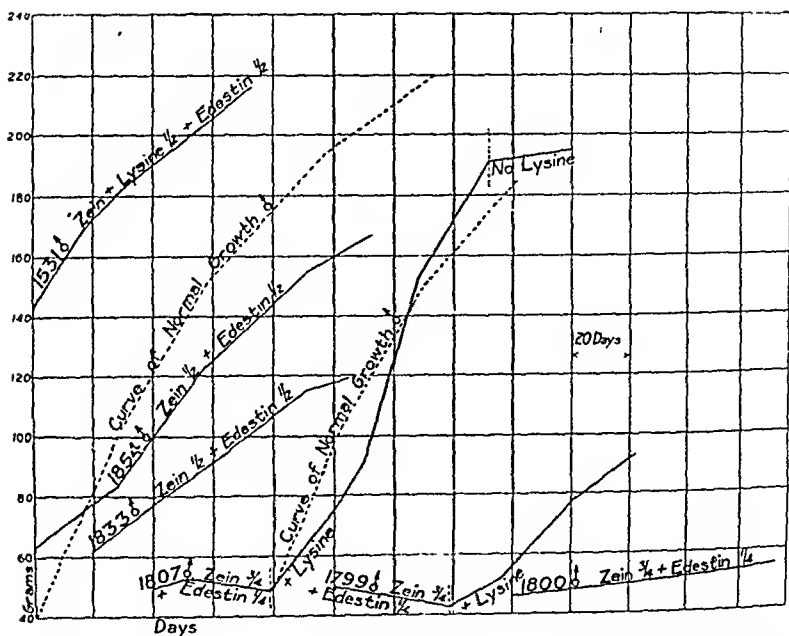


CHART II. RELATIVE PROPORTIONS OF PROTEINS YIELDING DIFFERENT AMOUNTS OF LYSINE WHICH PROMOTE NORMAL GROWTH WHEN THEY REPLACE A PART OF THE ZEIN. Rat 1160 shows growth at a normal rate when one-fourth of the zein in the food is replaced by lactalbumin which yields over 8 per cent of lysine. Rats 1422, 1434, 1447 show growth at a normal rate when three-fourths of the zein in the food is replaced by edestin which yields 1.65 per cent of lysine. That smaller additions of edestin fail to promote normal growth is shown in Chart III.

The composition of the foods used was as follows:

	RAT 1160	RATS 1422, 1434, 1447.
	grams	grams
Zein.....	13.5	4.5
Lactalbumin.....	4.5	0.0
Edestin.....	0.0	13.5
Protein-free milk.....	28.0	28.0
Starch.....	27.0	26.0
Lard.....	27.0	28.0
Water.....	12 cc.	4 cc.



**CHART III. RÔLE OF LYSINE IN GROWTH.** Inasmuch as edestin yields relatively little lysine (1.65 per cent) considerable additions of this protein to our zein food are necessary to produce growth. Thus, when one-quarter of the zein is replaced by edestin no growth is obtained, unless lysine as such is also added, whereupon rapid growth results. See Rats 1799, 1800, 1807. When one-half of the zein is replaced, better growth obtains. See Rats 1833, 1854. When lysine is added to a food containing equal parts of zein and edestin, practically normal growth results. See Rat 1531. Normal growth is obtained by a three-quarter replacement with edestin. (See Chart II, Rats 1422, 1434, 1447.)

The foods consisted of:

	RATS 1531, 1854.	RATS 1709*, 1800, 1807*.	RAT 1531.
	grams	grams	grams
Zein.....	9.0	13.5	8.70
Edestin.....	9.0	4.5	9.00
Lysine .....	0.0	0.0	0.54
Protein-free milk.....	28.0	23.0	28.00
Starch .....	21.5	25.7	24.50
Butter-fat.....	18.0	18.0	18.00
Lard.....	11.5	10.3	11.50
Water.....	7 cc.	12 cc.	7 cc.

\* These rats received food containing an addition of lysine equivalent to 3 per cent of the zein, as indicated on the chart.



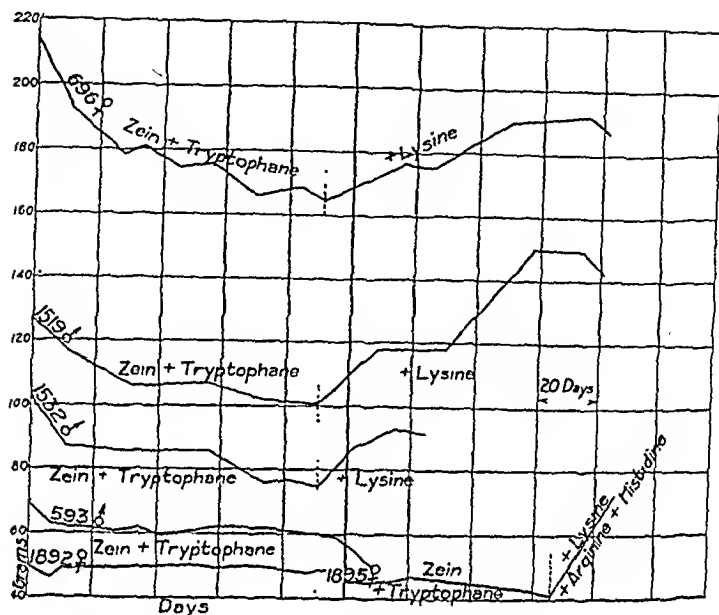


CHART V. INDISPENSABILITY OF TRYPTOPHANE FOR MAINTENANCE IN NUTRITION. These experiments should be contrasted with the failure of maintenance on zein-food alone, shown in Chart IV. Rats 696, 1519, 1532 show comparatively slow decline and tendency to maintenance when tryptophane replaced 3 per cent of the zein in the food. Rats 593, 1892, 1895 were almost perfectly maintained on similar foods for comparatively long periods. Rats 1892, 1895 received butter-fat in place of a part of the lard in their diet. The effect of further additions of lysine equivalent to 3 per cent of the zein, in promoting recovery or growth is seen in Rats 696, 1519, 1532, 1895. Further instances may be seen in Chart VI.

The foods contained:

	RATS 696*		RATS 1510, * 1532, * 593.		RATS 1892, 1895.*	
	grams		grams		grams	
Zein.....	17.46		17.46		17.46	
Tryptophane.....	0.54		0.54		0.54	
Protein-free milk.....	28.00		28.00		28.00	
Starch.....	27.00		27.00		27.00	
Butter-fat.....	0.00		0.00		18.00	
Lard.....	27.00		27.00		9.00	
Water.....	15 cc.		15 cc.		15 cc.	

\* Lysine equal to 3 per cent of the zein was added to the diet during part of the time as indicated in the chart. Rat 1895 also received an addition of arginine equivalent to 1.5 per cent and histidine equivalent to 1 per cent of the zein.

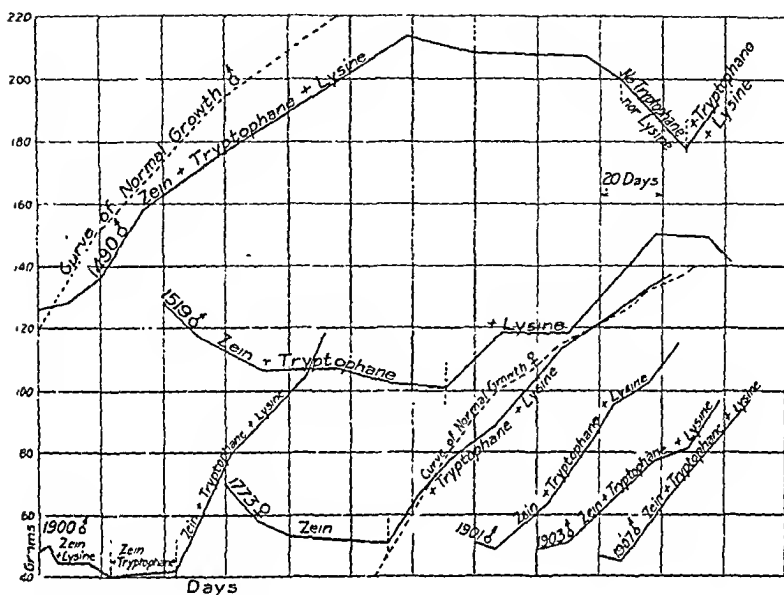


CHART VI. GROWTH ON FOODS CONTAINING ZEIN+TRYPTOPHANE+LYSINE.

The food contained:

	grams
Zein.....	16.92
Tryptophane.....	0.54
Lysine.....	0.54
Protein-free milk.....	28.00
Starch.....	27.00
Butter-fat.....	18.00
Lard.....	9.00
Water.....	15 cc.

The growth obtained on this diet may be contrasted with maintenance without growth in the absence of the lysine (see Chart V) and failure to be maintained in the absence of both lysine and tryptophane (Chart IV), thus demonstrating the rôle of these amino-acids in growth and maintenance respectively. That lysine cannot replace tryptophane in maintenance is shown by Rat 1900.

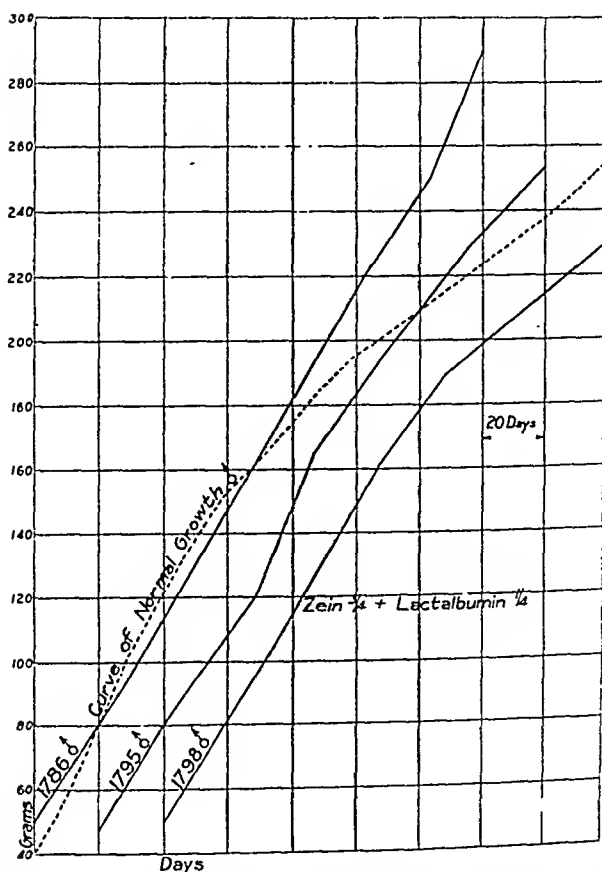


CHART VII. GROWTH ON FOOD IN WHICH ONE-QUARTER OF THE ZEIN IS REPLACED BY LACTALBUMIN.

The food consisted of:

	grams
Zein.....	13.5
Lactalbumin.....	4.5
Protein-free milk.....	28.0
Starch.....	27.3
Butter-fat.....	18.0
Lard.....	8.7
Water.....	12 cc.

The proportion of lactalbumin here represented obviously furnished sufficient lysine and tryptophane to permit growth at an approximately normal rate. Compare failures with corresponding additions of casein (Chart VIII) or of edestin (Chart III).

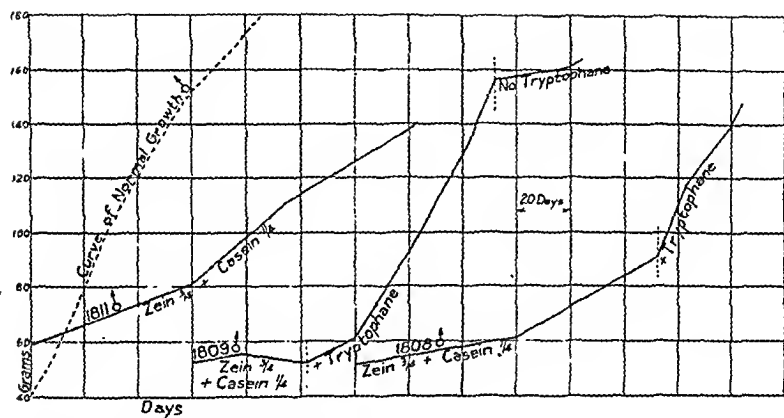


CHART VIII. EFFECT ON GROWTH OF REPLACING ONE-QUARTER OF THE ZEIN IN OUR FOODS WITH CASEIN.

The foods contained (prior to the addition of tryptophane equivalent to 3 per cent of the zein):

	grams
Zein.....	13.5
Casein.....	4.5
Protein-free milk .....	28.0
Starch.....	28.5
Butter-fat.....	18.0
Lard.....	8.5
Water.....	12 cc.

The inadequacy of this mixture for satisfactory growth must be compared with the success attending the use of the same proportion of lactalbumin (Chart VII). That this failure of the same proportion of casein to supplement the imperfect zein is due to its relative deficiency in tryptophane is demonstrated by the prompt renewal of growth when this amino-acid is added, and the cessation of growth when it is again omitted.



## ON SOY BEAN UREASE: THE EFFECT OF DILUTION, ACIDS, ALKALIES AND ETHYL ALCOHOL.

By E. K. MARSHALL, JR.

(From the Laboratory of Physiological Chemistry, Johns Hopkins University.)

(Received for publication, February 16, 1914.)

The conversion of urea into ammonium carbonate with the urease<sup>1</sup> of the soy bean has proven of value not only in urea determinations but in cases where urea interferes with some chemical manipulation since its removal can be readily accomplished by this enzyme.<sup>2</sup> Data relative to the action and behavior of urease under various conditions should prove of value in many connections.<sup>3</sup>

In our original paper on the use of urease in urea estimations, it was stated in discussing the time necessary for the complete decomposition of the urea in urine, that "The time required for the complete hydrolysis of the urea depends upon the quantity of urine used, the concentration of the urea, the amount of the enzyme present, and the temperature of the action. The velocity of the reaction is approximately twice as rapid at 35° as at 25° and directly proportional to the enzyme concentration within certain limits." The experimental data for this statement are presented in part in the present communication.

In order to determine the best conditions for the hydrolysis of urea by urease, the effect of various factors such as dilution, reaction of the medium, and enzyme concentration have been investigated.

<sup>1</sup> This *Journal*, xi, p. 283, 1913; xv, p. 487, 1913; xv, p. 495, 1913.

<sup>2</sup> Van Slyke: this *Journal*, xvi, p. 128, 1913; Abel, Rowntree and Turner: *Journ. of Pharm. and Exp. Ther.*, v, p. 315, 1914.

<sup>3</sup> A paper entitled, "A Study of the Mode of Action of Soy Bean Urease" was presented by Van Slyke and Cullen at the December meeting of the American Society of Biological Chemists.

Urease has received relatively very little study. Takeuchi and Armstrong are apparently the only ones who have worked with this enzyme of the soy bean.<sup>4</sup>

Takeuchi<sup>5</sup> submitted urease to the action of various substances before adding it to urea, and tested the activity of the solution after neutralization. One per cent  $\text{H}_2\text{SO}_4$ , 1 per cent  $\text{HCl}$ , 5 per cent  $(\text{NH}_4)_2\text{SO}_4$ , 0.05 per cent  $\text{CuSO}_4$ , 0.25 per cent  $\text{NaF}$  and 0.05 per cent  $\text{HgCl}_2$  were considered inhibitory to some extent, while 1 per cent  $\text{NaOH}$ , 2 per cent  $\text{H}_2\text{SO}_4$ , 10 per cent  $(\text{NH}_4)_2\text{SO}_4$ , 0.1 per cent  $\text{CuSO}_4$ , and 0.1 per cent  $\text{HgCl}_2$  were strongly inhibitory. His method however was unreliable consisting in an estimation of the length of time necessary for the development of a pink color with phenolphthalein. Armstrong and Horton,<sup>6</sup> and Armstrong, Benjamin and Horton<sup>7</sup> found that the ammonium carbonate formed in the reaction inhibits the velocity of decomposition of the urea but not nearly as markedly as ammonia, while carbon dioxide accelerated the rate of change. A few salts and non-electrolytes were studied in regard to their accelerating or inhibitory effect. Strong acids completely arrested the action, while amino-acids and other weak acids seemed to accelerate it. Ethyl alcohol in small amounts was found to exercise only a slight inhibitory effect.

#### EXPERIMENTAL.

The enzyme solution used in the experiments was prepared as described in former communications. Unless otherwise stated this extract prepared by digesting 1 part of ground soy beans with 10 parts of water, adding one-tenth volume  $\frac{N}{16}$   $\text{HCl}$  and filtering was used in all the experiments. The solutions were allowed to react in a thermostat at  $35^\circ$ . The reactions were interrupted at appropriate times by pouring the mixture into an excess of  $\frac{N}{16}$  hydrochloric acid, which destroys the enzyme practically instantaneously. The solution was then titrated with alkali and methyl orange to neutrality. No attempt was made to keep the enzyme concentration of the extracts the same in all experiments. The values given in the tables have been corrected in all cases for the alkalinity of the soy bean extract and for any acidity or alkalinity of the original solution. They represent the amounts of  $\frac{N}{16}$  hydrochloric acid required to neutralize the ammonium carbonate formed.

<sup>4</sup> For a collection of literature on the occurrence of urease in other connections see this *Journal*, xiv, p. 283, 1913.

<sup>5</sup> *Journ. of the College of Agriculture*, Tokyo, i, p. 1, 1909.

<sup>6</sup> *Proc. Roy. Soc.*, B, lxxxv, p. 109, 1912.

<sup>7</sup> *Ibid.*, lxxxvi, p. 328, 1913.

*Effect of enzyme concentration.*

In order to determine if the velocity of the reaction is directly proportional to the amount of enzyme used, series of urea solutions were allowed to digest with varying amounts of enzyme for periods of time which were inversely proportional to the enzyme concentration. This procedure eliminates any disturbing influence which the products of the reaction may exert, as the reaction proceeds in all cases to the same per cent completion. The total volume in all cases was 15 cc. Tables I and II indicate that the velocity of the reaction is practically proportional to the enzyme concentration, within the limits investigated.

TABLE I.

CONCENTRATION ENZYME	TIME	10 CC. 1 PER CENT UREA		10 CC. 0.5 PER CENT UREA	
		cc. $\frac{N}{10}$ HCl required	Per cent urea decomposed	cc. $\frac{N}{10}$ HCl required	Per cent urea decomposed
cc.	min.				
5	16.0	17.76	53.3	15.45	92.7
4	20.0	17.73	53.2	15.52	93.1
3	26.6	17.53	52.6	15.68	94.1
2	40.0	17.27	52.0	15.64	94.0
1	80.0	17.07	51.2	15.47	92.9

TABLE II.

CONCENTRATION ENZYME	TIME	10 CC. 1 PER CENT UREA		10 CC. 1.5 PER CENT UREA	
		cc. $\frac{N}{10}$ HCl required	Per cent urea decomposed	cc. $\frac{N}{10}$ HCl required	Per cent urea decomposed
cc.	min.				
5	20.0	31.80	95.4	33.55	67.1
4	25.0	31.35	94.0	33.00	66.0
3	33.3	31.05	93.1	32.40	64.8
2	50.0	30.43	91.3	31.90	63.8
1	100.0	29.25	88.0		

*Effect of dilution.*

The velocity of the decomposition of urea by urease increases with dilution to a maximum when with further dilution it decreases slightly. A tentative explanation of this phenomenon may be

ascribed to two factors: first, the urea and enzyme probably form an intermediate compound which is the actual substance undergoing decomposition, and second, the ammonium carbonate formed in the reaction retards its progress. On account of the first, dilution would tend to decrease the velocity by decreasing the amount of intermediate compound present, while on account of the second, it would increase the velocity by decreasing the concentration of hydroxyl ions from the ammonium carbonate.

TABLE III.

Five cc. 2 per cent urea solution + 2 cc. soy bean extract. Thirty minutes at 35°.

WATER ADDED	CC. $\frac{N}{10}$ HCL REQUIRED	PER CENT UREA DECOMPOSED
cc.		
0	10.66	31.9
5	11.74	35.2
10	12.76	38.3
20	13.62	40.9
30	14.48	43.4
40	14.79	44.4
50	14.94	45.0
60	15.14	45.4
70	15.27	45.8
80	15.30	45.9
90	15.19	45.6

TABLE IV.

Five cc. 2 per cent urea solution and 2 cc. soy bean extract. Sixty minutes at 35°.

WATER ADDED	CC. $\frac{N}{10}$ HCL REQUIRED	PER CENT UREA DECOMPOSED
cc.		
0	18.63	55.9
20	24.48	73.4
40	26.24	78.8
60	26.94	80.8
80	27.22	81.7
100	27.11	81.3
120	26.98	80.9
140	26.60	80.0

TABLE V.

Five cc. 1 per cent urea solution and 2 cc. soy bean extract.

AMOUNT WATER ADDED	30 MINUTES		60 MINUTES	
	cc. $\frac{N}{10}$ HCl required	Per cent urea decomposed	cc. $\frac{N}{10}$ HCl required	Per cent urea decomposed
cc.				
0	10.19	61.1	16.13	96.8
10	11.68	70.1	16.51	99.1
20	12.60	75.6	16.60	99.6
40	12.86	77.2	16.55	99.3
60	12.63	75.8	16.53	99.2
80	12.52	75.1	16.44	98.7
100	12.21	73.3	16.40	98.4
120	11.72	70.3	16.30	97.8
140	11.30	67.9	16.07	96.4

The following experiment shows the effect of dilution on the time necessary for the complete decomposition of the urea solution with different amounts of enzyme. Five-cc. portions of a 2 per cent urea solution were allowed to digest at room temperature for different time intervals with varying amounts of the soy bean extract. Similar solutions were prepared but diluted to 100 cc. and allowed to digest under the same conditions.

TABLE VI.

AMOUNT EXTRACT	TIME	UNDILUTED		DILUTED	
		cc. $\frac{N}{10}$ HCl required	Per cent urea decomposed	cc. $\frac{N}{10}$ HCl required	Per cent urea decomposed
cc.	hrs.				
0.5	4	10.47	31.4	15.19	45.6
0.5	22	26.89	80.6	33.15	99.4
0.5	47	31.79	95.7	33.20	99.6
1	4	17.68	53.0	28.98	86.9
1	22	33.16	99.4	33.13	99.4
1	47	32.00	96.0	33.05	99.1
2	4	29.55	88.6	33.05	99.1
2	22	32.55	97.7	33.25	99.7
2	47	32.05	96.2	33.05	99.1
3	4	32.88	98.5	33.07	99.2
3	22	32.68	97.9	33.23	99.7
3	47	32.10	96.4	33.04	99.1

*Effect of acid and alkali.*

As was emphasized in previous papers on the application of urease to the determination of urea in urine and blood, a very small concentration of hydrogen ions completely arrests the action of the urease. Accurate measurements of the hydrogen or hydroxyl ion concentrations in the reacting mixtures were not attempted for two reasons; the reacting mixture is constantly increasing in hydroxyl ion concentration as the decomposition of the urea progresses, and the protein present in the enzyme extract renders the procedure somewhat uncertain. A study, however, of the effect of varying amounts of acid and alkali on the rate of hydrolysis yields considerable information. The results given in the following tables show that the velocity of the reaction is not appreciably affected by changes of the hydrogen or hydroxyl ion concentration within rather narrow limits. An increase in the amount of either acid or alkali above these limits first retards the reaction, and finally when sufficient acid or alkali is present the enzyme ceases to act. A destruction of the enzyme also occurs when sufficient acid or alkali is present, and this is much more rapidly effected by acid than by the same concentration of alkali.

The reaction of the enzyme solution used has been determined as follows. Two-cc. portions of the extract were diluted to 10 cc. and titrated with  $\frac{N}{10}$  HCl and  $\frac{N}{10}$  NaOH and various indicators.

INDICATOR	CC. $\frac{N}{10}$ HCL REQUIRED	CC. $\frac{N}{10}$ NaOH REQUIRED	HYDROGEN ION CONCENTRA- TION FOR INDICATOR
Methyl orange.....	0.45		$10^{-4}$
Sodium alizarin sulphonate.....	0.15		$10^{-6}$
Rosolic acid.....		0.10	$10^{-7}$
Azolitmin.....		0.10	$10^{-7}$
Neutral red.....		0.20	$10^{-8}$
Phenolphthalein.....		0.30	$10^{-9}$

Five cc. of hydrochloric acid or sodium hydroxide were mixed with 2 cc. of enzyme solution, the mixture diluted to 10 cc. and the reaction tested.

CONC. HCl IN 5 CC. ADDED	CONC. NaOH IN 5 CC. ADDED	REACTION
0.000	0.000N	Alkaline to methyl orange, alkaline to alizarin, acid to rosolic acid.
	0.0006N	Neutral to rosolic acid.
	0.0015N	Alkaline to rosolic acid.
	0.003N	Alkaline to rosolic acid.
0.0009N		Alkaline to alizarin.
0.0015N		Alkaline to alizarin.
0.003N		Neutral to alizarin, alkaline to methyl orange.
0.006N		Acid to alizarin, alkaline to methyl orange.
0.009N		Acid to alizarin, about neutral to methyl orange.
0.015N		Acid to methyl orange.

The figures in the two tables given above show that the soy bean extract acts to some extent as a "buffer solution." Consequently, the concentrations of the hydroxyl and hydrogen ions as calculated from the concentrations of the hydrochloric acid or sodium hydroxide used are too large, and in reality must be considerably smaller. This explains the fact, as seen from the tables, that the smaller the amount of enzyme solution, the less the concentration of acid which is necessary to completely inhibit the reaction. A solution reacting neutral or only faintly acid or alkaline to rosolic acid possesses the proper reaction for the maximum decomposition of urea by the urease. In using the enzyme for decomposing urea, a solution should be neutralized to rosolic acid or litmus. A fluid which is slightly alkaline to methyl orange or sodium alizarin sulphonate but acid to phenolphthalein contains about the proper concentration of hydrogen or hydroxyl ions.

The experiments given in the table below were carried out as follows.

Five cc. of 0.3 M urea solution (1.8 per cent) were mixed with 5 cc. of the acid or alkali, placed in the bath at 35°, and treated with 5 cc. of a solution of the enzyme which had been previously warmed to 35°. The solution of the enzyme unless otherwise stated was made from the "usual extract" by diluting 40 cc. to 100 cc., 5 cc. being equivalent to 2 cc. of the original extract. The concentration of the various constituents of the mixture are expressed after mixing the solution, that is, when 5 cc. of 0.3 M urea solution and 5 cc. of 0.06 N acid were mixed, the concentration is expressed in the tables as  $\frac{M}{10}$  urea and 0.02 N acid, since the final volume in

all cases was 15 cc. The solutions were allowed to react at 35° for a definite period, then the action stopped as usual with HCl and the ammonium carbonate formed estimated. Different fresh preparations of the extract were used in each series, the same in any one series.

TABLE VII.

CONC. HCL	Enzyme, 2 cc. t, 40 min.	Enzyme, 2 cc. t, 60 min.	Enzyme, 1 cc. t, 60 min.
	cc. $\frac{N}{10}$ HCL REQUIRED		
0.000	15.80	21.80	12.46
0.0001N	16.08	21.67	12.22
0.0002N	16.12	21.82	12.61
0.0003N	16.02	21.90	12.38
0.0005N	16.08	21.60	12.46
0.0008N	16.04	21.77	12.43
0.001N	15.73	21.09	11.84
0.002N	13.57	17.22	8.61
0.003N	11.93	14.13	0.11
0.005N	0.00	0.00	0.00
0.01N	0.00	0.00	0.00
0.02N	0.00	0.00	0.00

TABLE VIII.

CONC. NaOH	Enzyme, 2 cc. t, 60 min.	Enzyme, 1 cc. t, 60 min.
	cc. $\frac{N}{10}$ HCL REQUIRED	
0.000	24.35	13.66
0.0001N	24.24	13.43
0.0002N	24.42	13.51
0.0003N	24.29	13.52
0.0005N	24.26	13.41
0.0008N	24.33	13.33
0.001N	24.09	13.27
0.002N	23.55	12.52
0.003N	23.00	11.72
0.005N	21.27	8.82
0.01N	11.13	1.60
0.02N	0.00	0.00
0.03N	0.00	0.00

TABLE IX.

Enzyme, 2 cc.; Time, 30 minutes.

CONCENTRATION $\text{NH}_3$	$\frac{N}{10}$ HCl REQUIRED
0.000N	17.67
0.002N	17.44
0.01N	15.86
0.03N	13.31
0.05N	9.72
0.1N	5.85
0.2N	2.55

The following data show that the enzyme is not only inhibited in its action but destroyed by sufficient strength of hydrochloric acid or sodium hydroxide. The experiments were performed as follows: 5 cc. of the dilute extract were treated with 5 cc. of hydrochloric acid or sodium hydroxide and allowed to stand for various lengths of time at room temperature. The mixtures were then made neutral to rosolic acid, warmed to  $35^\circ$  and treated with 5 cc. of 0.03 M urea solution and allowed to react for sixty minutes.

TABLE X.

Time of action of acid	2 min.	30 min.	18 hours
conc. HCl IN 5 cc. ADDED	cc. $\frac{N}{10}$ HCl REQUIRED		
0.000N	23.47	23.34	22.66
0.003N	22.27	21.15	17.85
0.006N	22.10	21.10	7.70
0.009N	19.50	8.32	0.66
0.015N	0.00	0.00	0.00

TABLE XI.

Time of action of alkali	2 min.	30 min.	18 hours
conc. NaOH IN 5 cc. ADDED	cc. $\frac{N}{10}$ HCl REQUIRED		
0.000N	24.35	24.54	23.65
0.003N	23.84	23.78	23.50
0.006N	22.78	22.98	22.96
0.009N	22.47	22.27	19.00
0.015N	20.66	19.16	5.00

*Effect of ethyl alcohol on urease.*

Small amounts of alcohol (20 to 30 per cent) do not appreciably retard the reaction, while urease continues to decompose urea in a medium of 80 per cent alcohol by volume. The experiments with alcohol were conducted as follows. Five cc. of 2 per cent urea solution were diluted to 95 cc. with alcohol and water, warmed to 35°, and treated with 1 cc. of the soy bean extract previously diluted to 5 cc.

TABLE XII.

Five cc. 2 per cent urea and 1 cc. soy bean extract. Thirty minutes at 35°.

PERCENTAGE ALCOHOL BY VOLUME	CC. $\frac{N}{10}$ HCL REQUIRED	PER CENT UREA DECOMPOSED
0	7.70	23.1
10	7.60	22.8
20	7.23	21.7
30	6.95	20.8
40	6.32	19.0
60	5.15	15.5
80	2.30	6.9

On standing with alcohol apparently very little destruction of the enzyme occurs. This is shown in the following table in which the experiments were conducted as follows: One cc. of soy bean extract was mixed with 9 cc. of alcohol and water so as to make a percentage of alcohol of the desired strength. The tubes were then allowed to stand at room temperature for two hours and

TABLE XIII.

Time of action of alcohol	2 hours	22 hours.
PERCENTAGE ALCOHOL BY VOLUME	CC. $\frac{N}{10}$ HCL REQUIRED	
0	15.85	14.69
10	15.80	14.75
20	15.70	14.48
30	15.62	14.26
40	15.39	13.62
60	15.40	13.04
80	15.38	12.41
90	15.16	12.75

twenty-two hours. The mixtures were diluted to 95 cc., warmed to 35°, treated with 5 cc. of a 2 per cent urea solution, and allowed to react for sixty minutes. The strongest alcohol in which the reaction occurred was therefore less than 10 per cent, which exerts practically no inhibitory effect.

#### CONCLUSIONS.

1. The velocity of the hydrolysis of urea by urease is practically proportional to the enzyme concentration.

2. The velocity of the hydrolysis increases with dilution to a maximum, and with further dilution decreases slightly.

3. The velocity of the hydrolysis is independent of the hydrogen or hydroxyl ion concentration within rather narrow limits.

4. Hydrochloric acid or sodium hydroxide in sufficient amounts inhibit the action of the enzyme and also destroy it.

5. Ethyl alcohol exercises only a moderate inhibitory effect, and its destructive action is apparently very slight.



## NOTE ON THE CONVERSION OF CREATINE TO CREATININE.

By FRANCIS G. BENEDICT.

(From the Nutrition Laboratory of the Carnegie Institution of Washington, Boston, Mass.)

(Received for publication, February 18, 1914.)

While carrying out a series of experiments in creatine metabolism at Wesleyan University in 1906-1907, Prof. Victor Caryl Myers, then a post-graduate student working under my direction, made the interesting and valuable suggestion that the conversion of creatine to creatinine might be most advantageously made at a high temperature. The autoclave method was then elaborated and demonstrated to be successful.<sup>1</sup> In preparing a series of papers to appear simultaneously, and as a result of the fact that being in Europe I did not see the proofsheets, the dual authorship was inadvertently retained in this article. Although I have spoken freely of the fact to visitors to this laboratory and especially to workers in many foreign laboratories, the method is proving so valuable, I feel constrained to make in print this tardy statement that the method should be known as the Myers method and I specially request that my name be no longer used in citing the method.

<sup>1</sup> *Amer. Journ. of Physiol.*, xviii, p, 397, 1907.



## THE INFLUENCE OF INULIN ON THE OUTPUT OF GLUCOSE IN PHLORHIZIN DIABETES.

By HOWARD B. LEWIS AND EDWARD M. FRANKEL.

(From the Department of Physiological Chemistry of the University of Pennsylvania, Philadelphia, Pa.)

(Received for publication, February 20, 1914.)

The question of the value of inulin as a significant source of energy for the organism has frequently been raised in connection with its use in diabetic dietaries. In a former paper by one<sup>1</sup> of us it has been shown that after the administration *per os* of large amounts of inulin, no significant quantities of inulin are to be detected in the feces. It is suggested that any utilization of inulin must result from hydrolysis to levulose in the stomach by the acid gastric secretion, a hydrolysis which in normal individuals can continue only through a short period, after which the acid of the gastric juice is neutralized in the intestine and its hydrolytic activity destroyed. Marked intestinal fermentation is observed to follow the ingestion of inulin, a fermentation which yields no levulose. Hence the opportunity for conversion to levulose is limited to the comparatively short period during which the acidity of the gastric secretion is active, since no specific enzymes for the hydrolysis of inulin exist in the mammalian gastrointestinal tract.

Recently Goudberg<sup>2</sup> has obtained similar results from feeding experiments with inulin and has also noted slight rises in the respiratory quotient in man after the administration of inulin. He also holds that the normal conversion of inulin to levulose in the stomach must be slight and believes that the increased respiratory quotients obtained by him are the result of oxidation of organic acids resulting from the bacterial fermentation of inulin.

<sup>1</sup> Lewis: *Journ. of the Amer. Med. Assoc.*, lviii, p. 1176, 1912, contains a review of the literature.

<sup>2</sup> Goudberg: *Zeitschr. f. exp. Path. u. Ther.*, xiii, p. 310, 1913.

## 366 Influence of Inulin on Glucose Elimination

In the present investigation a study of the influence of inulin feeding on the elimination of glucose in the urine of phlorhizinized dogs was undertaken. Since, as has been shown by Lusk and others,<sup>3</sup> levulose yields large amounts of glucose in phlorhizinized animals, it was believed that any significant conversion of inulin to levulose either in the stomach or elsewhere would be manifested by a rise in the glucose output in the urine. The methods used were those commonly employed in this laboratory.<sup>4</sup> The inulin used was a Kahlbaum preparation purified by precipitation with alcohol from an aqueous solution and did not reduce alkaline copper solutions. The levulose was a pure crystallized preparation of Merck. In each experiment an amount of levulose equivalent to the inulin fed previously was administered in order to secure a check on the power of each animal to eliminate as glucose any levulose absorbed from the alimentary tract.

As was anticipated, no significant amounts of extra glucose were eliminated after the feeding of inulin. In only one animal was there observed the elimination of any extra glucose, and that amount was so slight as to lie well within the limits of the normal variations. In every experiment the feeding of an equivalent amount of levulose led to the elimination of large quantities of extra glucose. The urines of the experimental periods of Dogs A and C were examined for levulose by the method of Pinoff<sup>5</sup> as modified by Taylor and Miller.<sup>6</sup> A weakly positive test was obtained with the urine of Dog C, period V (levulose) only.

### SUMMARY.

Inulin administered to phlorhizinized dogs gives rise to no glucose. The feeding of levulose to the same animals results in the elimination of large amounts of glucose. Since levulose administered to phlorhizinized dogs is largely excreted as glucose and not burned there seems little probability that an appreciable amount of inulin is converted to levulose or to any substance which can give rise to glucose in the diabetic organism.

<sup>3</sup> Cf. Lusk: *Ergeb. d. Physiol.*, xii, p. 375, 1912.

<sup>4</sup> Ringer, Frankel and Jonas: *this Journal*, xiv, p. 525, 1913.

<sup>5</sup> Pinoff: *Ber. d. d. chem. Gesellsch.*, xxxviii, p. 3308, 1905.

<sup>6</sup> Unpublished data.

TABLE I.

DATE 1914	PERIOD	WEIGHT kgms.	TOTAL NITROGEN grams	TOTAL GLUCOSE grams	D:N	EXTRA GLUCOSE grams	REMARKS
Dog A.							
Feb. 1	X	7.82	4.47	11.95	2.68	8.0	{ 9 gms. inulin per os. 10 gms. levu- lose per os.
2	XI	7.77	5.37	11.60	2.16		
2	XII		4.26	10.35	2.43		
3	XIII	7.47	3.63	17.70	4.88		
3	XIV		3.49	10.10	2.90		
Dog B.							
12	IX	12.52	9.25	34.70	3.75	1.9	{ 15 gms. inu- lin per os. 16.6 gms. levu- lose per os.
12	X		9.35	33.80	3.62		
13	XI		8.30	32.60	3.92		
13	XII	8.36	31.60	3.78	8.7		
14	XIII	7.39	35.60	4.82			
14	XIV	11.88	7.35	27.20	3.70		
Dog C.							
16	I	10.05	5.00	17.05	3.42	10.85	{ 18 gms. inu- lin per os. 20 gms. levu- lose per os.
16	II	9.24	4.45	14.65	3.30		
17	III		4.89	16.75	3.43		
17	IV		4.52	15.45	3.42		
18	V		4.40	26.45	6.02		
18	VI	4.81	17.70	3.68			



## THE NON-ANTIGENIC PROPERTIES OF RACEMIZED EGG ALBUMIN.

By CARL TEN BROECK.

(From the Department of Comparative Pathology, Harvard Medical School.)

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In 1907, Vaughan and Wheeler<sup>1</sup> formulated the theory that the introduction of a protein into the tissues of the body caused enzymes to be formed which destroyed and removed this foreign substance and these enzymes which are produced in excess are what we know as antibodies. Heilner<sup>2</sup> in the same year came to practically the same conclusions only he considers that it is the destruction of the protein which causes the antibodies normally present to be produced in excess. The latter is now the generally accepted opinion for it seems that the more easily a protein is broken down the more readily are antibodies produced. The albumins act as more powerful antigens than the compound proteins; digestion mixtures that do not give the protein reactions, though they still contain the constituents of the protein, fail to sensitize guinea pigs either to the mixture or to the original protein. The ease with which a substance is broken down is not the only factor which determines its antigenic properties. It must be a protein and, therefore, a colloid. Other factors of which we are more or less uncertain are also necessary. Wells<sup>3</sup> has pointed out that gelatin which contains most of the amino-acids, except tyrosine and tryptophane, though easily broken down by enzymes, fails to act as an antigen.

In the racemized proteins described by Dakin<sup>4</sup> we have sub-

<sup>1</sup> Vaughan, Victor C., and Wheeler, Sybil M.: *Journ. of Inf. Dis.*, iv, 1907.

<sup>2</sup> Heilner, E.: *Zeitschr. f. Biol.*, i, pp. 26-37, 1907.

<sup>3</sup> Wells, H. G.: *Journ. of Inf. Dis.*, v, pp. 449-483, 1908.

<sup>4</sup> Dakin, H. D.: this *Journal*, xiii, pp. 357-362, 1912; Dakin and Dudley: *ibid.*, xv, pp. 263-269, and 271-276, 1913.

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stances which in every way resemble simple proteins except for their diminished optical activity and the very remarkable property of being resistant to proteolytic enzymes and of not being metabolized when fed to or injected under the skin of experimental animals. At Dr. Dakin's suggestions the following experiments were undertaken to see whether these substances would act as antigens and thus throw light on the question as to whether a breaking down of the protein is necessary for antibody production.

Some preliminary experiments were made to sensitize guinea pigs with racemized casein but owing to the difficulty of getting a good sensitization with natural casein these experiments were given up and Dr. Dakin very kindly prepared some racemized egg albumin on which he gives the following notes concerning its preparation and properties.

A 20 per cent solution of Merek's soluble egg albumin in  $\frac{N}{2}$  sodium hydroxide was incubated at 37° for three weeks. During this time the optical rotation gradually fell until it reached a constant value. The racemized protein, after neutralization with dilute sulphuric acid, was then salted out by saturating the solution with ammonium sulphate. The precipitate was filtered off, suspended in a little water and dialyzed in the presence of a little toluene to free it from salts. The racemized egg albumin went into solution in the dialysis tube and was precipitated from this solution by the addition of alcohol.

The racemized egg albumin so obtained was filtered off and dried, and formed a white powder resembling the original protein. It gives the typical protein reactions (biuret, etc.) and differs chemically from the original substance only in its optical properties. Its aqueous solution coagulates on heating, as in the case of ordinary egg white. The reactions for tyrosine, tryptophane and cystine are all positive. Like racemized casein and caseose it is unaffected by the proteolytic enzymes of the digestive tract.

The first experiment was an attempt to sensitize guinea pigs by means of the racemized egg albumin either towards the latter or towards native egg albumin and also to see whether egg albumin would sensitize toward the racemized substance. The results are very clear-cut and are shown in Table I.

The two control animals, 990 and 995, after a seven weeks' interval were given intra-abdominal injections of 0.05 gram of the racemized egg albumin. Neither showed symptoms of anaphylaxis, and on the next day both were given the same amount

TABLE I.

GUINEA PIG		SENSITIZING INJECTION OF 0.005 GM. INTRA- ABDOMINAL	INTER- VAL IN DAYS	WEIGHT IN GRAMS	INTOXICATING INJECTION OF 0.05 GM. INTRA- ABDOMINAL	RESULTS
No.	Weight in grams					
968	600	Merck's egg albumin	20	670	Merck's egg albumin	Death in 2 hours. Distention of lungs and haemorrhages into stomach.
967	570	Merck's egg albumin	20	630	Racemized egg albumin	No symptoms.*
970	620	Racemized egg albumin	20	660	Racemized egg albumin	No symptoms.
969	560	Racemized egg albumin	20	625	Merck's egg albumin	No symptoms.
990	—	—		535	Merck's egg albumin	No symptoms ex- cept loss in weight.
995	—	—		580	Racemized egg albumin	No symptoms ex- cept loss in weight.

\* Twenty-four hours later an intravenous injection of 0.033 gram of egg albumin caused death in three minutes with a typical picture of anaphylaxis on autopsy.

of Merck's egg albumin. The animal that had previously received the egg albumin died in one hour, while the other was sick but showed no sneezing or "bucking" and it did not have the characteristic drop in temperature. The next day it was normal.

We must conclude from these experiments that racemized egg albumin even in relatively large amounts<sup>5</sup> does not sensitize guinea pigs toward itself or toward egg albumin and that egg albumin does not sensitize towards its optically inactive form. In guinea pigs sensitized to egg albumin the injection of the racemized substance does not produce an anti-anaphylactic state.

In order to study the complement binding and precipitin reactions two rabbits from the same litter were selected. One was given intra-abdominal injections of 0.05, 0.1, and 0.3 gram of the

<sup>5</sup> Wells, H. G.: *loc. cit.* finds that for 300-gram guinea pigs the minimal sensitizing dose of unpurified albumin is 0.0000063 gram while 0.00063 to 0.0000315 gram sensitizes so that nineteen days later 0.0025 gram injected into the peritoneal cavity produces fatal results.

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racemized egg albumin, at six-day intervals. The other rabbit was treated in the same way with Merck's egg albumin. Both animals showed a loss in weight, following the first two injections, which was quickly regained. Eight days after the last injection they were bled from the ear veins and the sera tested for specific precipitins, the results of the tests are given in Table II.

TABLE II.

NO.	SERUM OF RABBIT INJECTED WITH	AMOUNT OF SERUM PER CC. IN EACH TUBE	ANTIGEN USED	GRAMS OF ANTIGEN PER CC. IN EACH TUBE WITH RESULTS OF TEST				
				0.005	0.0025	0.00125	.000625	.0003125
624	Egg albumin	0.1	Racemized egg albumin	0	0	0	0	0
624	Egg albumin	0.1	Merck's egg albumin	++	++	+	+	+
623	Racemized egg albumin	0.1	Racemized egg albumin	0	0	0	0	0
623	Racemized egg albumin	0.1	Merck's egg albumin	0	0	0	0	0
632	(control rabbit)	0.1	Racemized egg albumin	0	0	0	0	0
632	(control rabbit)	0.1	Merck's egg albumin	0	0	0	0	0

In order to obtain a clear solution of the antigens it was necessary to dissolve them in distilled water. The racemized substance gave a clear solution, but the one containing egg albumin had to be clarified by filtration hence the amounts of the substance given in the table are somewhat high.

Eighteen days from the last injection these rabbits were again bled and their sera tested for complement fixing bodies. Titration of the haemolytic amboceptor showed complete haemolysis when 0.00075 cc. was used and almost complete with 0.0005 cc. In the titration of the antigen the racemized egg albumin in amounts of 0.001 gram showed a slight binding so that half this amount was used throughout with decreasing amounts of serum. At this time the egg-albumin serum seemed to have lost some of its precipitin for 0.0005 gram of egg albumin mixed with 0.1 cc. of serum failed to cause precipitation. The results are shown in

TABLE III.

TOTAL VOLUME 5 CC. INCUBATED 1 HOUR IN A WATER BATH AT 37° THEN PLACED IN REFRIGERATOR OVER NIGHT				HAEMOLYSIS AFTER TUBES HAD STOOD IN THE REFRIGERATOR OVER NIGHT		
TOTAL VOLUME 3 CC. INCUBATED 30 MIN. IN A WATER BATH AT 37°. HAEMOLYTIC SYSTEM THEN ADDED						
Rabbit No.	Inactivated Serum		Antigen in grams	Complement 1:10 dilution	Sheep's corpuscles 5% suspension	Amboceptor 1:1000 dilution
	Injected with	Amounts				
624	Egg albumin	0.2	0.0005	cc. 1	cc. 1	Using egg albumin as an antigen
624	Egg albumin	0.1	0.0005	1	1	0
624	Egg albumin	0.05	0.0005	1	1	0
624	Egg albumin	0.025	0.0005	1	1	50%
624	Egg albumin	0.01	0.0005	1	1	C
624	Egg albumin	0.2	0	1	1	C
624	Egg albumin	0.2	0	0	1	C
624	Egg albumin	0.2	0	1	0	0
624	Egg albumin	0.05	0	1	1	C
624	Egg albumin	0.05	0	1	0	C
623	Racemized egg albumin	0.2	0.0005	1	1	50%
623	Racemized egg albumin	0.1	0.0005	1	1	C
623	Racemized egg albumin	0.05	0.0005	1	1	C
623	Racemized egg albumin	0.025	0.0005	1	1	C
623	Racemized egg albumin	0.01	0.0005	1	1	C
623	Racemized egg albumin	0.2	0	1	1	C
623	Racemized egg albumin	0.2	0	0	1	0



Table III and study of them will show that with egg albumin as an antigen, there was complete binding of the complement with 0.1 cc. of the anti-egg-albumin serum, while there was no fixation when either serum was mixed with the racemized egg albumin or when the serum of the rabbit receiving the racemized egg albumin was mixed with the egg albumin.

It is not surprising that the racemized egg albumin fails to produce antibodies against the native egg albumin for Obermayer and Pick<sup>6</sup> have shown that proteins modified by the addition of various substances produce little or no precipitin against the original material, though Wells<sup>7</sup> did not obtain as conclusive results with iodized serum or egg albumin when he tested antibody production by means of the anaphylactic reaction. These modified proteins do, however, produce precipitin against themselves, and as far as I have been able to learn the above experiments are the first in which a substance having the reaction of a simple protein has failed to act as an antigen.

Support is, therefore, given to the theory that it is the breaking down in the tissues, of an injected protein that causes the production of antibodies.

<sup>6</sup> Obermayer and Pick: *Wien. klin. Wochenschr.*, xiv, p. 327, 1906.

<sup>7</sup> Wells, H. G.: *loc. cit.*



## A METHOD FOR THE DETERMINATION OF FAT IN SMALL AMOUNTS OF BLOOD.

By W. R. BLOOR.

(From the Laboratory of Biological Chemistry, Washington University,  
St. Louis, Mo.)

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The fats do not lend themselves readily to chemical change, consequently the only methods for their quantitative determination in use at the present time are those which depend on the direct weighing of the material extracted by means of various fat solvents, with or without previous destruction of the tissue. An excellent discussion of the various methods for fat determination in tissues is given by Leathes<sup>1</sup> so that it is not thought necessary to enter into the discussion in this paper.

The most recent and at present the most widely used of these methods is that ordinarily called the Kumagawa-Suto<sup>2</sup> method, a modification of the Liebermann procedure, in which the tissue is destroyed by heating with strong alkali, and the fatty material extracted from the resulting solution, purified and weighed. This method gives very good results with most tissues except blood, with which a special modification is necessary. In the Shimidzu<sup>3</sup> modification, the blood is first extracted with alcohol, the blood residue and the extract then submitted separately to hydrolysis with alkali, after which both are extracted and the extracts purified and weighed as before. This method requires several hours' time and a considerable volume of blood—ordinarily 25 cc. Some practice is required to get satisfactory results and even then the final petroleum-ether extract often contains color which it is hard to remove. Moreover, there is always the possibility that the long and severe treatment that the fats undergo during the separa-

<sup>1</sup> Leathes: *The Fats*, p. 55.

<sup>2</sup> Kumagawa-Suto: *Biochem. Zeitschr.*, viii, p. 212, 1908.

<sup>3</sup> Shimidzu: *ibid.*, xxviii, p. 237, 1910.

tion, and during which air is not excluded, may produce changes in certain of the more sensitive fats which render them insoluble in the final solvent or destroy them altogether. There is always a suspicion that the resinous, colored substance, soluble in ether, but insoluble in petroleum ether, contains some material which should properly be classified as fat. It is known<sup>4</sup> that fats of the liver, kidney, and heart undergo change (oxidation) on exposure to air, and I have found the same to be true of blood fats. A sample of blood fatty acids, completely soluble in petroleum ether, after standing in an open dish in the laboratory for about two weeks, darkened in color and was then only 80 per cent soluble in petroleum ether. The remaining sticky mass was readily soluble in cold dry ether. It is quite possible that some such change takes place in the fat during the separation by the Kumagawa-Suto method.

A method was sought which would give satisfactory results in a shorter time and with a much smaller amount of blood, such as might be drawn frequently from a vein without changing the normal metabolism—a method suitable for physiological investigations on fat metabolism involving examination of the fat content of the blood over a considerable period of time.

The method to be described depends on a new principle—the determination of the fat by precipitation in a water solution, and comparison of the cloudy suspension so obtained with that of a similarly prepared standard fat solution by the use of the nephelometer. The determination may be completed in about three-quarters of an hour and may be carried out with from 0.5 cc. to 5 cc. of blood. Ordinarily about 2 cc. are used. It has been found to be accurate to within 5 per cent of the total fat. The procedure is as follows:

*Extraction.* About 2 cc. of blood are drawn from the vein with a graduated syringe and run at once with stirring into a weighed graduated flask containing about 40 volumes of a mixture of 3 parts alcohol and 1 part ether. After again weighing to find the weight of blood added, the solution is raised to boiling in a water bath, cooled under the tap, made up to volume with the alcohol-ether, mixed and filtered. The filtrate is water-clear and almost colorless.

<sup>4</sup> Hartley: *Journ. of Physiol.*, xxxviii, p. 17.

*Determination.* From 5-20 cc. of the extract (containing about 2 mgm. of fat) are measured with a pipette into a small beaker and saponified by evaporating just to dryness with 2 cc. of  $\frac{N}{1}$  sodium ethylate. The dry residue is gently warmed with 5 cc. of the alcohol-ether until all but the flakes of alkali is dissolved, then poured slowly with stirring into 100 cc. of distilled water in a beaker and the saponification beaker well washed out with the water.

A similar solution of the standard is prepared by adding 5 cc. of the standard fat solution (see below) from a pipette with stirring to 100 cc. of distilled water. To the standard and to the test solutions are added simultaneously 10-cc. portions of dilute (1:4) hydrochloric acid and the solutions allowed to stand for five minutes, after which they are transferred to the comparison tubes of the nephelometer.

For the comparison, the two tubes, filled to the same height with the solutions, are placed in the nephelometer with the standard tube always on the same side. If bubbles appear on the walls of the tubes they should be removed by inverting the tubes two or three times. The movable jacket on the standard tube is set at a convenient point, generally 50 mm. (Richards' nephelometer) and comparisons made by adjusting the jacket on the test solution until the images of the two solutions show equal illumination. Not less than five readings are taken, alternately from above and below, and the average taken as the correct reading.

The standard solution used is an alcohol-ether solution of pure triolein of which 5 cc. contain about 2 mgm. of fat. The alcohol and ether used for the standard are freshly redistilled absolute alcohol and pure dry ether.

#### *Discussion of the procedure.*

*The extraction.* The above simple procedure has been found to give a good extraction of the fatty substances from blood. Running the freshly drawn blood slowly into the solvent which is kept in motion causes the precipitation of the blood proteins in a finely divided flocculent form—in excellent condition for extraction. The solvent combines the penetrating power of alcohol with the greater solvent power of the ether. Under these condi-

tions the presence of the great excess of solvent and the short heating is believed to be adequate to extract all but the most difficultly extractable fatty material.

No claim is made that the extraction is complete, for it can readily be shown that such is not the case. Continued boiling of the whole blood or of the corpuscles with the alcohol-ether for some time results in a greater yield of material, but the solution is colored reddish brown so that obviously more than fat is being dissolved. Similarly, treatment of the blood or of the washed corpuscles with saponin sufficient to produce haemolysis, results in an increased yield. On the other hand, neither continued boiling nor treatment with saponin changes the yield of fat from the serum or plasma. It is believed therefore that the above treatment extracts all the fat from the blood except what is contained in the interior of the corpuscles. As will be shown in a later publication, the method as used above has been found adequate to remove the fat completely from milk and forms the basis of a simple and rapid method for the determination of fat in milk. The procedure gives results within the limit of error of the nephelometer on duplicate samples of from 1-5 cc. of blood from various sources with and without the addition of emulsified fat. Since Iscovesco<sup>5</sup> has found that the lipid content of the corpuscles is fairly constant for the species, it is believed that the method is adequate to measure changes in the fat of the blood in normal and pathological conditions with a good degree of accuracy. In view of these facts it has not been thought desirable at the present time to use the above mentioned or other means for increasing the yield, since thereby the procedure is complicated without any certain advantage being attained.

*The saponification.* Sodium ethylate is used to ensure saponification of the cholesterol esters. Heating the saponification mixture after it has reached dryness generally does no harm, but in some cases, particularly where a small aliquot of the fat solution is used, too high results are sometimes obtained. The reason for the difference is not clear, but since the addition of a drop or two of water before the evaporation, prevents the increase in value, it is believed to be due to the action of the sodium on some constituent of the mixture.

<sup>5</sup> Iscovesco: *Compt. rend. soc. biol.*, i, p. 985, 1912.

*The standard.* The choice of triolein as the standard is empirical, triolein being readily obtainable of a sufficient degree of purity. It might be thought that a mixture of fats more nearly approximating that of the blood would be a more desirable standard, but since the composition of blood fat is variable, any standard which might be chosen would be open to objection for the same reason. Again, the question may be raised whether it is possible to compare a suspension of unsaponified triolein with that of the saponified blood fat as is done above. As will be shown below, such a comparison is possible and since the use of the unsaponified standard simplifies the procedure, it has been used throughout.

*Choice of time for comparison.* It was pointed out by Wells<sup>6</sup> with regard to solutions of this nature that since very small particles do not reflect (ordinary) light, the reflection must be due to aggregates of particles, and since also when aggregates pass a certain size the light reflected diminishes because of the decreased surface, the reflecting power must increase to a maximum and then diminish. In agreement with the above it has been found that both standard and test fat solutions change continuously in reflecting power and it is necessary to determine approximately the rates of change in the solutions so as to find out under what conditions accurate comparisons may be made. Accordingly, solutions of (a) unsaponified triolein, (b) saponified triolein, and (c) saponified blood fat, prepared as in the method and all of about the same strength, were studied in the nephelometer using for standard a ground glass plate. After mixing with the acid, readings were taken from time to time up to one hour. The results obtained are expressed in the diagram (p. 382). Each of the curves is the average of several closely agreeing determinations.

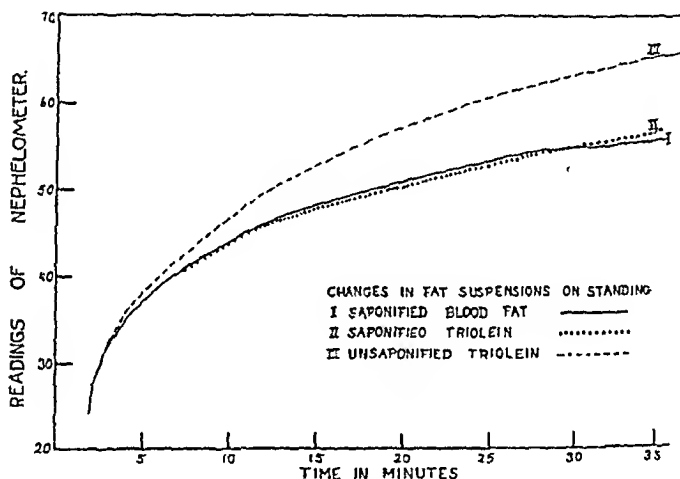
It will be noticed from the curves:

1. That the maximum, if any, is attained very quickly—before the first readings could be made.
2. That all the solutions decrease in value during the observations, rapidly during the first five minutes and then more slowly. (The changes continue slowly until in the course of some days all the fat has separated out.)
3. That the rate of decrease in value of the triolein solution

<sup>6</sup> Wells: *Amer. Chem. Journ.*, xxxv, p. 99.

is more rapid than that of either of the soap solutions, the rate of change in which is practically the same during the time of observation.

4. That the differences in the readings of the triolein solution and the soap solutions are within the error of the instrument (2-3 mm. for the single reading) up to ten minutes. The procedure in the comparison of the solutions is based on these findings. The solutions are allowed to stand about five minutes to equalize differences in the time of addition of the precipitating



acid and the readings are then made during the succeeding five minutes.

*The nephelometer—Calibration.* A form<sup>7</sup> of the Richards' nephelometer<sup>8</sup> was used. It was found that the addition of a rack and pinion to the jacket on the comparison side was a great convenience. Matched test tubes were used for comparison tubes and as it was not found possible to find two tubes which when filled with the same solution, would give the same readings on both sides of the instrument, the tubes were marked and always used on the same

<sup>7</sup> Supplied by the International Instrument Company of Cambridge, Mass.

<sup>8</sup> Richards: *Zeitschr. f. anorg. Chem.*, viii, p. 269, 1895; Richards and Wells: *Amer. Chem. Journ.*, xxxi, p. 235, 1904.

side, the one used for the standard solution being on the left. The average of many readings of the right hand tube was then taken as the potential height of the standard.

Lacking a nephelometer, many colorimeters may be adapted for the purpose as has been shown by Kober<sup>9</sup> with the Duboscq colorimeter, and the author<sup>10</sup> with the Schreiner instrument.

Since the amount of light reflected is not exactly proportional to the depths of solution (because of different distances of the reflecting surfaces from the eye, absorption of light by the upper layers of liquid, etc.), it is necessary to calibrate the nephelometer for different strengths of test solution, and for different standards, if more than one is used. The values obtained are plotted on a curve and corrections in the readings made from it.

Again, since the total light reflected from a given depth of solution depends as well on the size of the particles as on their number, it is obvious that success with a nephelometric method depends on securing as nearly as possible the same aggregation of the particles in the solutions at different times. Care must be taken therefore to secure the same chemical and physical conditions—as regards concentration of alcohol-ether, acid, etc.—also that the solutions to be compared should not be too far different in value. (Readings above 70 and below 30—with the standard at 50—are discarded.)

Correction must be made for reagents. By the use of freshly distilled alcohol and ether for the solvents and of carefully cleaned sodium and freshly distilled absolute alcohol for the ethylate, it has been possible to reduce the correction very considerably but not entirely to eliminate it. The necessary correction is most easily found by superposing an equal amount of the alcohol-ether and of the alkali on the amount of a blood-fat solution ordinarily used for a determination, then making the determination in the usual way. From the differences in value obtained the correction is easily calculated.

<sup>9</sup> Kober: this *Journal*, xiii, p. 485, 1913.

<sup>10</sup> Bloor: *Proceedings of the Amer. Chem. Soc.*, Rochester, 1913.

*Results.*

Duplicate determinations made on the same fat solution and also on different samples of blood taken at short intervals from the same animal agree with one another within the limits of error of the instrument (about 5 per cent). Fat added to the blood in the form of a casein emulsion may be recovered quantitatively, as may be seen from the results below. Soap or lecithin emulsions generally give too high results, probably because of the changes in corpuscles produced by these substances.

I. 2.24 gram of a casein emulsion<sup>11</sup> of olive oil containing 0.31 gram of fat were added to 51 grams of blood of which the fat content was 0.7 per cent.

Total fat present in the blood..... 0.68 gram.

Found by the nephelometric method..... 0.69 gram.

1.3 gram of a casein emulsion of olive oil containing 0.215 gram of fat were added to 15.4 gram of blood of which the fat content was 0.6 per cent.

Total fat present in the blood..... 0.30 gram.

Found by the nephelometric method..... 0.32 gram.

The results obtained by the nephelometric method on blood agree quite well with those obtained by the Kumagawa-Suto method, although somewhat higher throughout, as may be seen from the table.

	KUMAGAWA-SUTO METHOD	NEPHELO-METRIC METHOD
Beef blood (defibrinated slaughter house blood)	0.28	0.31
Dogs' blood. I. Normal.....	0.56	0.62
Dogs' blood. II. Fat young dog at the height of digestion.....	2.50	2.70
Dogs' blood. III. Fasting for several days.....	0.75	0.84
Pigs' blood (defibrinated slaughter house blood)	0.48	0.51
Hens' blood (very fat).....	0.93	0.97

The method is being used in the study of changes in the fat content of the blood under various conditions.

The preliminary work on this method was done in the Gordon Hall Laboratories of Chemistry of Queen's University, Kingston, Canada, and it is a pleasant duty to acknowledge the kindness of the director, Professor W. L. Goodwin, and of Dr. W. D. Bonner in placing the facilities of the laboratories at my disposal.

<sup>11</sup> Raper: this *Journal*, xiv, p. 117, 1913.

# THE COMPARATIVE CHEMISTRY OF MUSCLE: THE PARTITION OF NON-PROTEIN WATER-SOLUBLE NITROGEN.

By D. WRIGHT WILSON.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven, Connecticut.)

(Received for publication, March 2, 1914.)

Since Liebig (1847) developed a world-wide interest in the extract of beef, the study of the non-protein water-soluble constituents of muscle has continuously progressed. The compounds which have been identified are so diverse in character and unlike in chemical constitution that, for lack of a better classification, they have received the general name of "extractives." The methods developed by Kossel, Kutscher,<sup>1</sup> and their coworkers for the isolation and identification of the basic nitrogenous extractives have been the means of discovering many new compounds.

Different investigators, working on the extracts of muscles from various animals, have shown marked qualitative differences in the constituents. Where Kutscher (1905) and Gulewitsch (1900) and their pupils found creatine, creatinine, carnosine, methyl-guanidine, carnitine, etc., in beef extract, they isolated arginine, lysine, betaine, erangonine, etc., from crab extract (Ackermann and Kutscher, 1907). Chittenden (1875) obtained considerable quantities of glycocoll from the scallop, an invertebrate, whereas Miesko (1908) showed that the monoamino-acids are minor constituents of beef extract.

Knowledge of these large variations in the qualitative composition of extracts of muscles from various animals has led to increasing interest in the comparative study of the quantitative relations of the constituents. Liebig's (1847) classic experiment, in which a large amount of creatine was obtained from the tired muscles of a hunted fox, immediately stimulated work on muscle creatine and creatinine. The introduction of the colorimetric method by Folin<sup>2</sup> greatly expanded the field and showed that much

<sup>1</sup> See Abderhalden's *Handbuch der biochemischen Arbeitsmethoden*, II, p. 1044.

<sup>2</sup> Folin: *Zeitschr. f. physiol. Chem.*, xli, pp. 223-242, 1904.

of the previous work was incorrect. With this method, Mellanby (1908) made careful comparative studies on muscles from various animals. The work has recently been extended by Cabella (1913), Becker (1913) and Myers and Fine (1913), in whose papers a review of the literature on creatine will be found. The data there presented show a maximum value of 0.52 per cent creatine for the fresh striated muscle of the rabbit and a tendency toward decrease among the lower forms of vertebrates to minimal figures of 0.24 per cent and 0.25 per cent for the skate and lamprey. An exception is noted in the case of the hedge-hog, a hibernating animal with a muscle-creatine content of 0.2 per cent. Satisfactory evidence for creatine in invertebrates has not yet been presented.

After the work of Burian and Hall (1903), Scaffidi (1910-1912) made a comparative study of the "free" and "total" purines in various muscles. He reported a maximum value in the muscle of the duck and a minimum in the mantle of the polyp.

Many of the early investigations involved quantitative variations of the individual constituents but few attempts were made to determine the nitrogen partition of the non-protein water-soluble compounds in muscle. Soave (1906), König and Bömer (1895) and Baur and Barsehall (1906) showed that a large percentage of the nitrogen of the muscle extracts from higher vertebrates was precipitated by phosphotungstic acid. Since 1909, many investigators have approached the problem from different points of view. Extracts of various muscles, mainly from vertebrates, were studied by Suzuki, Joshimura, Jamakawa and Irie (1909), Skworzow (1910), v. Fürth and Schwarz (1910), Yoshimura (1911), Delaunay (1910), Folin and Denis (1912), Van Slyke and Meyer (1913) and Buglia and Constantino (1912). These investigations brought out, among other things, the importance of the compounds precipitated by phosphotungstic acid from the muscle extracts of the higher vertebrates; especially creatine and carnosine, the nitrogen of which at times comprised two-thirds of the total extractive nitrogen.

Part of the work of the last named investigators (1912) deserves more detailed consideration here. They made nitrogen partitions on the extracts of muscles from fourteen different species of animals comprising examples from various classes of vertebrates and invertebrates, ranging from the ox to the worm. Total extractive nitrogen and amino nitrogen (by Sørensen's formol titration method) in the total extract and the phosphotungstic acid filtrate were determined. Their data show the following interesting relations: The total extractive nitrogen increases from 0.37 per cent of the fresh muscle of the ox to over four times that value among the lower forms. The values for the total amino nitrogen increase in like manner from 0.059 per cent to 0.413 per cent. The increase is not gradual in descending the scale of animals, since great variations occur. For example, in *Scyllium catulus*, a cartilaginous fish, the extractive nitrogen is four and one-half times greater than in the ox, while the amino nitrogen is less. In general, the values for the total extractive nitrogen and the total free amino nitrogen are higher in the invertebrates. In the higher forms, there is usually an excess of diamino- over monoamino-acid nitrogen, whereas

among the invertebrates, the monoamino-acid nitrogen predominates. In this respect, the cartilaginous fishes resemble the invertebrates, among which, however, the octopus is an exception. High ammonia values after hydrolysis of the cartilaginous fish extracts supplement the observations of Städeler and Frericks (1858) and Schröder (1890) that large amounts of urea are present. It is interesting to note that the amount of monoamino-acid nitrogen found in the octopus extract corresponds almost exactly to the amount of taurine found by Henze (1905).

The isolation of betaine, taurine, glycocoll, arginine, lysine, etc., from invertebrates and cartilaginous fishes, together with the analytical findings already discussed, suggested a more thorough investigation of the nitrogen partition of the protein-free extracts of the muscles of molluscs and animals near the border line between vertebrates and invertebrates. Such a study might throw further light on the quantitative variations and possibly indicate desirable fields for qualitative examination. For this purpose, I have determined the total nitrogen and amino nitrogen in the total protein-free water extract and in the phosphotungstic acid filtrate of the same; also the total sulphur in the total extract, and the creatine wherever possible.

Muscles from the following animals were examined:

#### *Invertebrates.*

##### Mollusca.

Gastropoda—Periwinkle (*Sycotypus caniculatus*), fresh pedal muscle.

Lamellibranchiata—Clam (*Venus mercenaria*), the two adductor muscles and the foot (fresh).

Scallop (*Pecten irradians*), the fresh adductor muscle.

Cephalopoda—Squid (*Loligo pealii*), the mantle muscle preserved in alcohol for several months.

Arthropoda—*Limulus polyphemus*, the small muscle bundles from various parts of the body, preserved in alcohol for several months.

#### *Vertebrates.*

Cyclostomata—Lamprey (*Petromyzon marinus*), the body after the removal of the skin, fins, internal organs, head, notochord, etc., dried at 80°.

A modification of Van Slyke's method for the determination of amino nitrogen in the tissues<sup>3</sup> was employed. In general, the fresh or preserved muscle tissue was ground in a meat chopper,

<sup>3</sup> D. D. Van Slyke: this *Journal*, xvi, pp. 187-195, 1913.

carefully mixed, and a portion taken for a moisture determination. From 300 to 500 grams were heated on a water bath for one-half hour with 3-5 times their weight of water (made acid with 1 cc. of 50 per cent acetic acid per liter) with occasional stirring. The liquid was decanted through glass wool and the tissues again comminuted. The entire amount was reextracted from four to eight times with hot acidulated water on a water bath for periods of 15-20 minutes each. When a considerable amount of the finely divided particles ran through the glass wool, the extracts were filtered through paper pulp with suction. The combined extracts were concentrated under reduced pressure in Claissen flasks to about 150 cc. and the residual protein precipitated by adding five volumes of 95 per cent alcohol. Owing to their high content of glycogen which is also precipitated by the alcohol, the periwinkle, clam and scallop extracts gave large precipitates. After standing over night, the alcoholic solution was filtered and the precipitate washed with 80 per cent alcohol. The filtrate was made alkaline to phenolphthalein with sodium hydroxide and distilled under reduced pressure. The procedure removed the ammonia, present in so small amounts that the removal was doubtless unnecessary.<sup>4</sup> After most of the alcohol had been removed and the volume of fluid reduced to about 100 cc., water was added, the solution acidified with acetic acid and distillation continued. Another concentration was sometimes necessary to remove the last traces of the alcohol which interferes with the amino nitrogen determination.

The concentrated extract was washed from the distilling flask and made up to a definite volume, usually 250 cc. From three to 10 cc. were necessary for the amino nitrogen determinations by Van Slyke's method.<sup>5</sup> Total nitrogen was determined by the Kjeldahl method. For total sulphur, Denis's<sup>6</sup> modification of Benedict's method was used after evaporating the extract to dryness on a water bath with a little fuming nitric acid.<sup>7</sup> Before the barium chloride was added, the solution was boiled with a

<sup>4</sup> The ammonia nitrogen in the clam was 0.89 per cent and in the periwinkle, 0.98 per cent of the total extractive nitrogen.

<sup>5</sup> D. D. Van Slyke: this *Journal*, xii, pp. 275-284, 1912.

<sup>6</sup> W. Denis: this *Journal*, viii, pp. 401-403, 1910.

<sup>7</sup> C. G. L. Wolf and E. Osterberg: *Biochem. Zeitschr.*, xxix, pp. 429-438, 1910.

little alcohol<sup>8</sup> to remove any free chlorine which might be formed on adding the hydrochloric acid to the ignited residue. This seemed to improve the precipitation of the barium sulphate.

The phosphotungstic acid precipitation was varied slightly with the different extracts in order to obtain sufficient material for the amino nitrogen determinations. In general, to 100 cc. of the original extract, 10 grams of sulphuric acid (diluted somewhat) and an excess of a 50 per cent solution of phosphotungstic acid were added and the volume made up to 200 cc. with water. The flask was then heated in a boiling water bath and rotated constantly until most of the precipitate had dissolved and the remainder was finely granular. The flask was allowed to cool and stand for twenty-four hours to complete the precipitation. The solution was filtered through a dry filter paper and a portion neutralized with sodium hydroxide, then acidified with acetic acid and concentrated under reduced pressure to a volume small enough so that 10 cc. or less were sufficient for an amino nitrogen determination. Total nitrogen and amino nitrogen were estimated. An illustrative protocol follows:

*Limulus*. The small muscle bundles were removed from recently killed animals and placed in 95 per cent alcohol. Portions of the fresh muscle were dried at 110°C. to constant weight. Water: 82.5 per cent. Six months later, the alcohol was strained off and concentrated under reduced pressure. The tissues were ground fine and mixed thoroughly with the concentrated alcoholic solution. A sample was dried. Water: 76.3 per cent.

A portion, 227 grams, equivalent to 363 grams of fresh tissue, was extracted six times. The first extracts were light canary yellow and scarcely opaque, indicating that no large amount of glycogen was present. The combined extracts were concentrated under reduced pressure to 125 cc. and 625 cc. of 95 per cent alcohol added. After standing over night, the alcoholic solution was filtered and the small precipitate washed. The filtrate was made alkaline to phenolphthalein with sodium hydroxide solution and concentrated, acidified and concentrated again and made up to 250 cc.

*Amino nitrogen.*

VOL. USED	TIME OF REACTION	VOL. N GAS	VOL. CORRECTED FOR REAGENTS	VOL. CORRECTED FOR "AMINES," ETC.	BAR.	TEMP.	MG. N	N IN PER CENT OF FRESH MUSCLE
		cc.	cc.	cc.	mm.			
10 cc.	4 min.	16.1	15.7	15.5	764	21°	8.80	0.061

<sup>8</sup> A personal suggestion from Professor S. R. Benedict.

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TABLE 2.

*Nitrogen partition and sulphur in the non-protein water extract of muscle.*

	GRAMS NITROGEN PER 100 GRAMS FRESH MUSCLE			GRAMS NITROGEN PER 100 GRAMS DRY MUSCLE		
	Total extract	PWA filtrate Monoamino acid frac- tion	PWA ppt. Diamino acid frac- tion (by difference)	Total extract	PWA filtrate Monoamino acid frac- tion	PWA ppt. Diamino acid frac- tion (by difference)
<i>Total (Kjeldahl) nitrogen.</i>						
Lamprey.....	0.248	0.040	0.208	1.31	0.212	1.10
Limulus.....	0.441	0.076	0.365	2.98	0.514	2.46
Squid.....	0.425	0.190	0.235	1.78	0.799	0.99
Clam.....	0.558	0.306	0.252	2.14	1.18	0.97
Scallop.....	0.504	0.339	0.165	2.63	1.77	0.86
Periwinkle.....	0.541	0.167	0.374	1.94	0.598	1.34

<i>Amino nitrogen.</i>						
Lamprey.....	0.010	0.004	0.006	0.053	0.021	0.032
Limulus.....	0.061	0.031	0.030	0.412	0.209	0.203
Squid.....	0.151	0.127	0.024	0.635	0.534	0.101
Clam.....	0.285	0.266	0.019	1.10	1.02	0.073
Scallop.....	0.283	0.268	0.015	1.47	1.40	0.078
Periwinkle.....	0.146	0.118	0.028	0.524	0.423	0.100

<i>Non-amino nitrogen (by difference).</i>						
Lamprey.....	0.238	0.036	0.202	1.26	0.190	1.07
Limulus.....	0.380	0.045	0.335	2.57	0.304	2.26
Squid.....	0.274	0.063	0.211	1.15	0.265	0.89
Clam.....	0.273	0.040	0.233	1.05	0.154	0.90
Scallop.....	0.221	0.071	0.150	1.15	0.370	0.78
Periwinkle.....	0.395	0.049	0.346	1.42	0.176	1.24

<i>Total sulphur (expressed as grams S).</i>						
Lamprey.....	0.012			0.063		
Limulus.....	0.046			0.310		
Squid.....	0.165			0.694		
Clam.....	0.370			1.42		
Scallop.....	0.133			0.694		
Periwinkle.....	0.175			0.628		

TABLE 3.

*Amino and non-amino nitrogen in per cent of total extractive nitrogen.*

	AMINO NITROGEN			NON-AMINO NITROGEN		
	Total extract	PWA filtrate Monoamino acid fraction	PWA ppt. Diamino acid fraction	Total extract	PWA filtrate Monoamino acid fraction	PWA ppt. Diamino acid fraction
Lamprey.....	4.03	1.61	2.42	96.0	14.5	81.5
Limulus.....	13.8	7.04	6.80	86.2	10.5	76.0
Squid.....	35.6	29.9	5.65	64.4	14.7	49.7
Clam.....	51.1	47.7	3.41	48.9	7.2	41.7
Scallop.....	56.2	53.2	2.98	43.8	14.0	29.8
Periwinkle.....	27.0	21.8	5.18	73.0	9.0	64.0

extractive N ratio (44.9 per cent) observed by Buglia and Constantino.

*Non-amino nitrogen.* This shows much smaller differences and none of the characteristic variations observed in the amino nitrogen fraction. The values for the limulus and the periwinkle are higher than for the other animals and the lamprey no longer exhibits a minimal figure.

*Nitrogen in the monoamino- and diamino-acid fractions—See Table 2.*

The causes for the variations already discussed can be traced further by examination of the values for amino and non-amino nitrogen in the fractions produced by precipitation with phosphotungstic acid. In this way, we get a clue to the general character of the nitrogenous compounds in the various extracts.

*Amino nitrogen.* The amino nitrogen in the phosphotungstic acid filtrate—the monoamino-acid fraction—shows even greater variations than that of the total extract. It increases from 0.004 per cent in the lamprey to 0.268 per cent in the scallop. The amino nitrogen in the phosphotungstic acid precipitate—the diamino-acid fraction—shows no great variations except the small value for the lamprey. Only in the lamprey extract is the amino nitrogen in the diamino-acid fraction greater than that in the monoamino-acid fraction. This relation, together with the low absolute value, is characteristic of vertebrate muscle extracts. In the limulus, the two forms of amino nitrogen are about equal

in amount, but in the lower types of animals the monoamino-acid fraction is much the greater and, in the cases of the scallop and the clam, contains nine-tenths of the total amino nitrogen or over 50 per cent of the total extractive nitrogen.

This observation, suggesting the presence of an abundance of monoamino-acids, is interesting in view of the fact that Chittenden (1875) isolated glycocoll from the scallop and showed, for the first time, that amino-acids occur free in nature. Other compounds that may be present, inasmuch as they have been isolated from related forms, are leucine, tyrosine, alanine (Ackermann and Kutscher, 1907; Suzuki, et al., 1909, 1912) and taurine<sup>10</sup> (Mendel, 1904; Henze, 1905; Mendel and Bradley, 1906) which contribute all their nitrogen to the amino nitrogen of the monoamino-acid fraction.

The values for amino nitrogen in the diamino-acid fraction show no great variations except the low figure for the lamprey. The maximum value, which occurs in the limulus extract, is only 0.03 per cent of fresh muscle or less than 7 per cent of the total extractive nitrogen. These figures show the slight importance, in point of quantity, of this fraction of the muscle extracts. The compounds from which it may be derived will be discussed below.

*Non-amino nitrogen.* Most of this is in the phosphotungstic acid precipitate and usually comprises more than nine-tenths of all the nitrogen therein. The periwinkle extract shows the maximum figure. This may be accounted for, at least in part, by the presence of betaine which I isolated in considerable amounts from this tissue (Wilson, 1913). Betaine undoubtedly forms a part of this fraction in all these types of animals, for, in addition to the above case, it has been isolated from the following animals: Mussel, *Mytilus edulis* (Brieger, 1885; Jansen, 1913), octopus (Henze, 1910), dogfish (Suva, 1909), a cephalopod (Suzuki, et al., 1909, see

<sup>10</sup> As no data for the quantitative determination of the amino nitrogen in taurine by Van Slyke's method were found in the literature, the following experiment was performed, showing that the amino nitrogen in taurine reacts quantitatively with nitrous acid in a manner similar to  $\alpha$ -amino nitrogen. The taurine used was an analyzed specimen isolated from the abalone, *Haliotis*, (Mendel 1904), and kindly furnished by Professor Mendel.

Taurine used, 0.1016 gram containing 0.0114 gram N. Obtained 0.0116 gram N. The reaction was complete in five minutes.

Kutscher, 1909), crab extract (Ackermann and Kutscher, 1907), scallop (Wilson, 1913).

Among other compounds isolated, histidine, arginine, lysine, carnosine, creatinine and other less well known substances (Suzuki, et al., 1909, 1912; Ackermann and Kutscher, 1907), if present, contribute to the diamino-acid fraction. Some of these contain an  $\alpha$ -amino group which reacts with nitrous acid and appears as amino nitrogen. For this reason, one-third of the histidine N, one-fourth of the arginine N, 95 per cent of the lysine N and probably one-fourth of the carnosine N would appear in the amino nitrogen fraction. The  $\omega$ -amino group in lysine reacts at a slower rate than the  $\alpha$ -amino group, but most of it comes off in the regular reaction time. The remainder of the nitrogen in these compounds and all the nitrogen in creatinine and betaine would appear as non-amino nitrogen in the diamino acid fraction. If much urea were present, it would be partially precipitated by the phosphotungstic acid and recorded here.

An indication of the presence of urea in lamprey extract was obtained in the determinations of the amino nitrogen. The volumes of nitrogen gas obtained from a portion of the total extract in different reaction periods are given in the table below.

VOL. USED	TIME OF REACTION	VOL. N	INCREASE IN N VOL.	INCREASE IN 4-MIN. PERIODS	TEMP.
	min.	cc.	cc.	cc.	
10 cc.	4	6.4			21°
	9	8.4	2.0	1.6	
	12	9.7	1.3	1.7	
	16½	10.9	1.2	1.1	

The above data show that, after the  $\alpha$ -amino groups had been completely decomposed, some compound or compounds were present which reacted slowly with nitrous acid giving about the same amount of nitrogen in similar intervals of time. Van Slyke has shown that urea, ammonia, amines and amino purines are slowly attacked under these conditions. Urea reacts with nitrous acid at a slow and fairly uniform rate and is completely decomposed only after about eight hours. The ammonia was removed by the previous distillation with sodium hydroxide. Of the remaining compounds,

urea has been shown to be present in large amounts in cartilaginous fishes (Schröder, 1890; Städeler and Frerichs, 1858) and it is undoubtedly the main cause for the results noted above. The phosphotungstic acid filtrate gave the normal reaction: in five minutes, 2.6 cc. of N, in fifteen minutes, 2.7 cc. of N. This indicates that, whatever the cause of the anomalous reaction with nitrous acid, it had been removed by the phosphotungstic acid.<sup>11</sup> The amount of amino nitrogen in the lamprey extract was so small that the material used (319 grams) failed to furnish enough for satisfactory determinations, but the indications of a very low content of amino nitrogen are, without doubt, valid.

The non-amino nitrogen in the phosphotungstic acid filtrate is small in amount in each extract and shows no characteristic variations. It may be derived, in part, from compounds like urea, creatine and proline which have been reported as constituents of similar muscle extracts. Some of the nitrogen undoubtedly comes from compounds like urea which are incompletely precipitated with phosphotungstic acid by the method used.

#### *Further considerations.*

The *total sulphur* (see table 2) in the total extract was determined in order to shed some light on the possible presence of taurine which is known to occur in the periwinkle and the scallop. The figures range from a maximum of 0.370 gram of S in the extract from 100 grams of fresh muscle of the clam to a minimum of 0.012 gram of S for the lamprey.

A *creatine* determination in the lamprey extract showed the presence of 0.24 per cent creatine in fresh muscle or 1.25 per cent in dry muscle, which is slightly lower than Mellanby's finding of 0.25 per cent. Creatine has actually been isolated from lamprey muscle by Wilson and Lyman (1913). The colorimetric determination on the limulus extract, outlined in the protocol, is hardly to be taken as conclusive in view of the facts that the color reactions were not entirely characteristic and creatine has never been isolated from the muscles of invertebrates. The observed reaction is interesting, however, as showing the presence of some sub-

<sup>11</sup> The phosphotungstic acid used, Kahlbaum's, precipitated urea from an acid solution.

stance not present in the other invertebrates examined. No trace of a similar color reaction with Jaffé's test was apparent in the muscle extract of the squid.

*Amino nitrogen* was determined *before and after hydrolysis* of portions of the total extract (Table 1). The increase after hydrolysis indicates the presence of amino-acid combinations, probably in the form of low polypeptides, as they were not precipitated by 80 per cent alcohol.

#### SUMMARY.

A nitrogen partition was made in extracts of muscles from the lamprey, limulus, squid, clam, scallop and periwinkle. Total nitrogen (Kjeldahl) and amino nitrogen (Van Slyke) were determined in the total protein-free extracts and in the filtrates from the phosphotungstic acid precipitations.

The lamprey muscle contained a minimum of total extractive nitrogen, most of which was non-amino nitrogen. The values for the total amino nitrogen rose from a minimum in the lamprey which was only 4 per cent of the total extractive nitrogen, to a maximum in the clam and scallop which was over 50 per cent of the total extractive nitrogen.

The major portion of the amino nitrogen in lamprey extract was precipitated by phosphotungstic acid. This is a characteristic of vertebrate muscle extracts. The amino nitrogen was equally divided in the limulus extract between the phosphotungstic acid precipitate and filtrate but, in the lower animal forms, the monoamino-acid fraction showed a great excess which, in the scallop, comprised 53 per cent of the total extractive nitrogen. This corresponds with the fact that glycocoll is readily isolated from that muscle.

The non-amino nitrogen is high in all the extracts examined. The major portion is in the diamino-acid fraction in which it greatly exceeds the amino nitrogen. Of the compounds isolated from these and similar extracts, betaine, urea, proline, arginine, histidine and creatine contribute all or part of their nitrogen to the non-amino nitrogen fraction. The various qualitative and quantitative relations indicate that betaine is probably an important constituent of extracts of muscles from these types of animals.

The amount of creatine in lamprey muscle confirms Mellanby's

finding and identifies this species in a chemical way with the vertebrates rather than the lower forms. The presence of urea is indicated.

Total sulphur was determined in the total extracts.

The great qualitative and quantitative variations between the extracts of muscles from different vertebrates and invertebrates suggest fundamental differences in the tissue metabolism of these animals. Why creatine, which is apparently an important constituent of muscle even among the lowest types of vertebrates, is absent from the most complicated forms of invertebrates studied, is an unsolved riddle. The significance of the change from the complex basic extractives of vertebrate muscle to the simpler amino-acids in the invertebrates is equally unintelligible. The similarity between the extractives of these low animal forms and of plants may be significant. The presence of betaine and the absence of creatine are characteristic of both. Comparative studies on the extractives of these simpler forms of life may throw some light on their rôle in the animal economy and aid in solving some of the problems of intermediary metabolism. Experiments like the above, besides furnishing interesting data, suggest points of attack where the variations are sufficiently large to furnish fruitful investigations.

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# THE INFLUENCE OF COD LIVER OIL AND SOME OTHER FATS ON GROWTH.<sup>1</sup>

BY THOMAS B. OSBORNE AND LAFAYETTE B. MENDEL,

WITH THE COÖPERATION OF EDNA L. FERRY AND ALFRED J. WAKEMAN.

(From the Laboratory of the Connecticut Agricultural Experiment Station and the Sheffield Laboratory of Physiological Chemistry in Yale University, New Haven, Connecticut.)

(Received for publication, March 5, 1914.)

The inability of young albino rats to complete their growth on a diet consisting of isolated proteins, starch, "protein-free milk" and commercial lard called attention to the need of some substance in supplementing the ordinary nutrients and enabling the characteristic increment in body-weight to proceed to its normal limits. The reasons for the conclusion that the lacking factor was something ordinarily found in the fat component of milk have been explained elsewhere.<sup>2</sup> In those communications the efficiency of butter in restoring growth in animals that had ceased or failed to grow on our mixtures of isolated food substances in which commercial lard furnished the fat component of the dietary was demonstrated. It was further shown that butter-fat prepared from the butter is quite as effective as the latter or as milk, in promoting the recovery and renewed growth of animals which have met with the nutritive disaster already described.

It has been our experience during the late summer and fall of the past three years that young rats on our protein-free milk diets almost invariably fail to grow, while at other seasons on the same diet they make normal growth for about three months. The failure to make early growth is accompanied by diarrhoea, diminished appetite and, after three or four weeks, by inflamed eyes, which soon develop into a purulent state. In a previous paper<sup>3</sup> we stated that the addition of butterfat to the diet speedily

<sup>1</sup> The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

<sup>2</sup> This *Journal*, xv, pp. 311-326, 1913; *ibid.*, xvi, pp. 423-437, 1913. See also McCollum and Davis: *ibid.*, xv, p. 167, 1913.

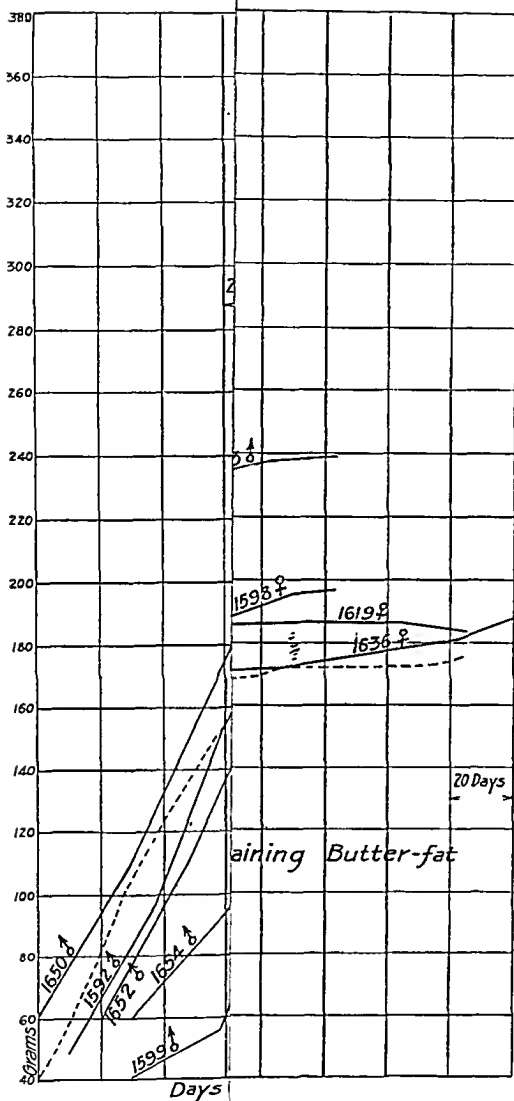
<sup>3</sup> Osborne and Mendel: this *Journal*, xvi, p. 431, 1913.

CHART I. Showing completed growth of rats on a diet consisting of

	<i>per cent</i>
Purified protein.....	18
Starch.....	26
Protein-free milk.....	23
Commercial lard..	10
Butter-fat.....	18

The proteins used were casein, edestin, or lactalbumin. The prolonged success of these feeding experiments is attributable to the *butter-fat* included in the diet. When the fat component is lard alone, young rats invariably fail to complete their normal growth, as already shown in a previous paper (this *Journal*, 1913, xvi, p. 423), in which the earlier growth of some of these animals was charted. In view of frequent nutritive failures on less perfect rations, it is worthy of note that the 14 records here presented include all of the animals started on this diet, with the exception of one which died very early from some unknown cause.

These are the first in all respects successful experiments made with growing animals on a diet of isolated food substances.



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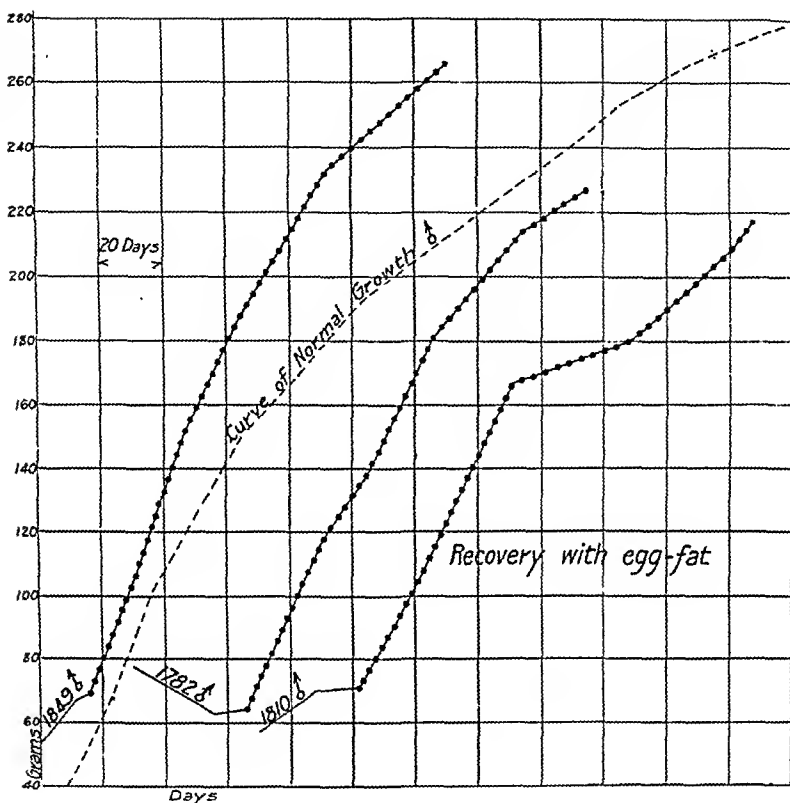


CHART II. Showing restoration of growth (rats 1782, 1810) or uninter-  
rupted growth (rat 1849) on a diet containing

	per cent
Purified protein .....	18
Starch.....	28
Protein-free milk .....	28
Commercial lard. ....	8
Egg yolk fat.....	18

in which the *egg yolk fat* appears to be as efficient as butter fat. (Cf. Chart I.)

The periods of egg-yolk-fat feeding are indicated by the interrupted line (- o - o - o).

CHART III. Showing the favorable influence of *cod liver oil* in promoting recovery and prolonged growth of rats on a diet in which either 6 per cent or 18 per cent of the lard is replaced by cod liver oil. Rats 1893 and 1898 received only 6 per cent of cod liver oil; the remainder received 18 per cent. The composition of the two diets was as follows:

	per cent	per cent
Purified protein.....	18	18
Starch.....	28	23
Protein-free milk.....	23	28
Commercial lard.....	20	8
Cod liver oil.....	6	18

The results may be compared with those in Charts I (butter-fat) and II (egg yolk fat).

The periods of cod liver oil feeding are indicated by the interrupted line (- o - o - o - o).

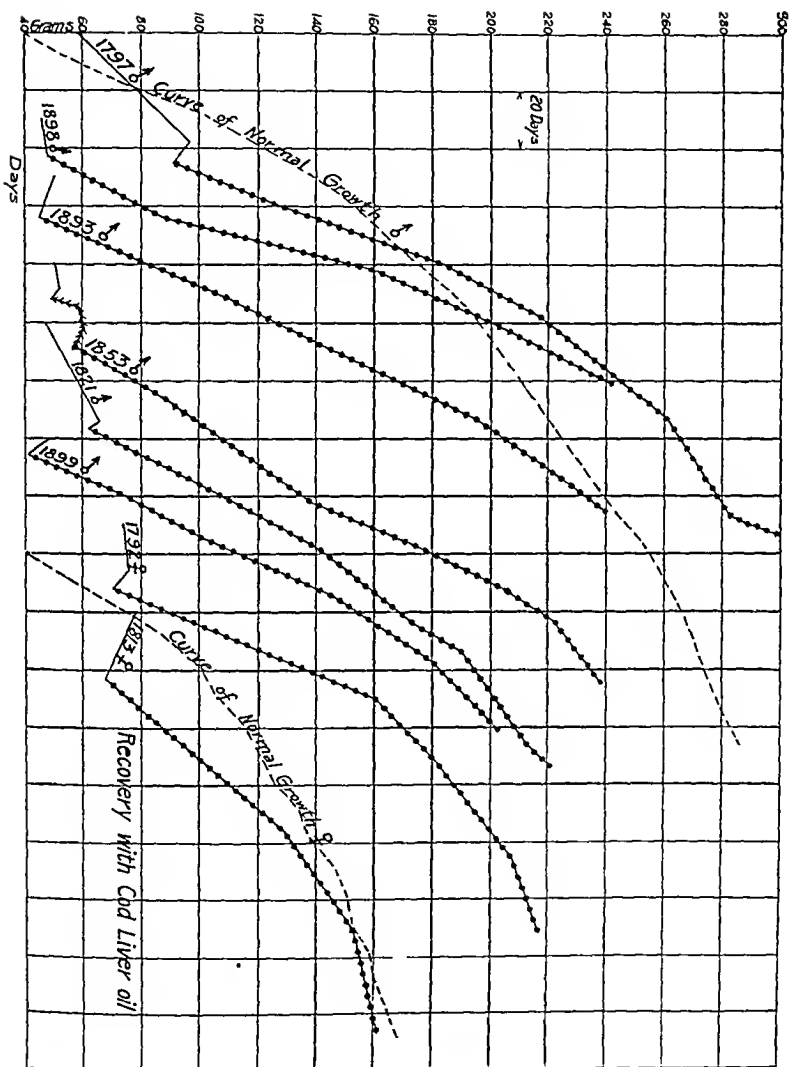


CHART III

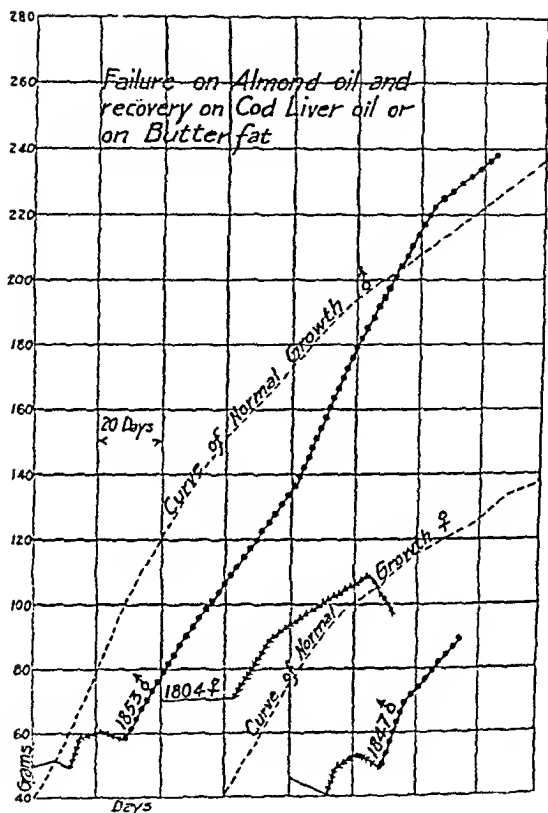


CHART IV. Showing the failure of *almond oil* to restore or promote growth in the same way as does butter-fat, egg yolk fat, or cod liver oil. The diet consisted of

	per cent
Edestin ..	18
Starch.....	28
Protein-free milk ..	28
Commercial lard ..	8
Almond oil.....	18

Note the prompt recovery when *cod liver oil* (rat 1853) or *butter-fat* (rat 1847) indicated by an interrupted line (- o - o - o) replaced the *almond oil* indicated by the interrupted line (←←←←←←←←←←).

# A SIMPLIFIED AND INEXPENSIVE OXIDASE APPARATUS.<sup>1</sup>

By HERBERT H. BUNZEL.

(From the Bureau of Plant Industry, U. S. Department of Agriculture,  
Office of Plant Physiological and Fermentation Investigations.)

(Received for publication, March 6, 1914.)

In 1912 the writer described an apparatus for the determination of oxidases in plant juices.<sup>2</sup> This has since proved very satisfactory and has made possible various investigations on the relation of oxidases to various plant diseases<sup>3</sup> and studies on the mechanism of oxidase activity.<sup>4</sup> The apparatus has not found its way into general use in plant physiological laboratories, probably on account of the great expense of manufacture. A simpler and less expensive apparatus, shown in the accompanying illustration, was therefore devised.

After manometer *M* is detached at the ground joint, a measured quantity of the plant juice is run into compartment *E*, and the oxidizable substance in solution into the compartment *F*. Enough water is then added to make the total volume of liquid in the apparatus 6 cc. The manometer is replaced in such a way that the openings *C* and *D* coincide, and the apparatus warmed to the temperature at which the experiment is to be carried out. To close the apparatus the manometer is turned in the ground joint through an angle of 90 degrees.

The plant juice and chromogenic substance do not mix until the apparatus is shaken. If solid substances are used instead of

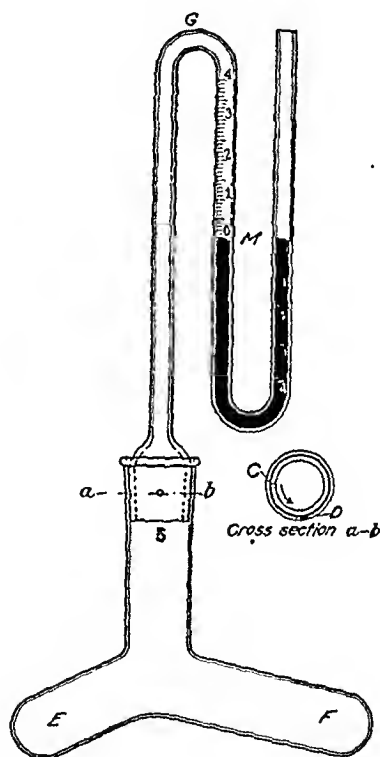
<sup>1</sup> Published by permission of the Secretary of Agriculture.

<sup>2</sup> Bunzel, Herbert H.: The Measurement of the Oxidase Content of Plant Juices, U. S. Department of Agriculture, Bureau of Plant Industry, Bulletin 238, 1912; Also, Ein neuer Apparat zur Bestimmung von Oxydasen in Gewebesäften, *Zeitschr. f. biol. Technik, u. Methodik.*, ii, pp. 307-309.

<sup>3</sup> Bunzel, Herbert H.: A Biochemical Study of the Curly-Top of Sugar Beets, U. S. Department of Agriculture, Bureau of Plant Industry, Bulletin 277, 1913; Bunzel, Herbert H.: Quantitative Oxidase Measurements, *Orig. Comm. Eighth Internat. Congr. of Applied Chem.*, xix, pp. 37-44.

<sup>4</sup> Bunzel, Herbert H.: Not yet published.

solutions, they are introduced through a funnel by means of a camel's-hair brush. The stem of the funnel is wider than that usually used for liquids and is slightly bent at its end in order to insure complete transfer of the substance into the compartment. When dry substances are used, the water is added separately afterwards by means of a pipette.



The volume of the apparatus up to point *G* on the manometre is 25 cc. When 6 cc. of liquid are used the volume of gas is 19 cc. Every change in pressure of 1 centimeter of mercury, therefore corresponds to the absorption of 0.25 cc. of oxygen. On account of the reduction of the gaseous volume in this apparatus it is much more sensitive than the other. In fact, it is so sensitive that 0.1 cc. of potato juice or 0.005 gram of dried buds of the tulip tree will give readings ranging from 0.5 to 2.0 centi-

meters when pyrocatechol or hydrochinon is used as the oxidizable substance.

In addition to its simplicity and increased sensitiveness, the apparatus is much less fragile and very much easier to clean than the old one. As its only drawback must be mentioned the fact that there is no provision made for absorption of the carbon dioxide produced. Until the carbon dioxide production in the oxidation of the various oxidase reagents has been determined, the apparatus will furnish only comparative results.

The apparatus may be secured from the Emil Greiner Company, 45 Cliff Street, New York City.



# ON THE BEHAVIOR OF EMULSIN IN THE PRESENCE OF COLLODION<sup>1</sup>

By ROY E. CLAUSEN.

(From the Rudolph Spreckels Physiological Laboratory of the University of California.)

(Received for publication, March 6, 1914.)

## I. INTRODUCTION.

### *a. Object of the investigation.*

Numerous investigators have observed that solid, neutral media, such as animal charcoal, are able to absorb, or adsorb, enzymes from their solutions.<sup>2</sup> These phenomena have been studied in some special cases to some degree of completeness, and it has been found that they are similar to other absorption phenomena; but certain difficulties peculiar to the study of enzyme processes make it difficult to establish these facts accurately. The conclusion, however, that the inactivation of the enzyme solutions when placed in contact with these absorption media is due to the absorption of the enzyme and its subsequent retention by the absorption medium has been generally accepted. Nevertheless, investigations on the effects of various artificial membranes, in particular collodion, seemed to show that in some cases the inactivation of the enzyme solutions could not be explained in this way, but presented notable differences.

Thus Miss Porter<sup>3</sup> found that collodion in the form of carefully prepared tubes rapidly inactivated all the enzymes studied with the exception of taka-diastrase. It was found impossible to recover more than a trace of the enzyme from the collodion films, and, moreover, there was no evidence of saturation of the membranes

<sup>1</sup> Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the University of California.

<sup>2</sup> Cf. Euler: *General Chemistry of the Enzymes*, p. 81, 1912.

<sup>3</sup> Porter: *Quarterly Journ. of Exp. Physiol.*, iii, p. 375, 1910.

on repeated treatment with the enzyme solutions. This, together with the inhibitive power which developed in most cases in the solutions as a result of inactivation, led to the conclusion that the inactivation was not due to simple absorption, but that the enzymes themselves were changed by contact with the membranes into substances having inhibitive powers. The work herein outlined on the behavior of emulsin solutions in the presence of collodion membranes was started, therefore, in order to determine the quantitative relations involved in these phenomena.

### *b. Interpretation of results.*

The lack of any direct means by which the enzyme content of a solution may be measured makes it necessary to employ the indirect method of measuring the activity of the solution. The interpretation of results from such data, however, is attended with difficulty on account of the irregularity of the behavior of enzymes both with respect to the rate at which they decompose the substrate and the proportionality which exists between enzyme concentration and measured activity. We are not concerned in this investigation with the factors which determine these relations, but merely with a means of conveniently and at the same time accurately discussing the data herein presented.

The time law which governs the hydrolysis of salicin by emulsin and the various factors which affect it have not yet been satisfactorily determined. Tammann<sup>4</sup> and Henri<sup>5</sup> have shown that for long time periods the values of the unimolecular constant decrease notably during the course of the reaction, and Henri<sup>6</sup> and Herzog<sup>7</sup> on the basis of these results, have proposed more complicated formulae to express the time relations. The first period of the reaction does not, however, give any regular deviations from the monomolecular time law. Thus calculations from Tammann's<sup>8</sup> earlier investigations in which the course of hydrolysis of a 2.7 per cent solution of salicin was followed by determining

<sup>4</sup> Tammann: *Zeitschr. f. physikal. Chem.*, xviii, p. 426, 1895.

<sup>5</sup> Henri: *Lois générales de l'action des diastases*, Paris, p. 102, 1903.

<sup>6</sup> *Loc. cit.*

<sup>7</sup> Herzog: *Konink. Akad. v. Wetensch. te Amsterdam, Sitzber.*, 1903; *Zeitschr. f. Physiol.*, iv, p. 163, 1904.

<sup>8</sup> Tammann: *Zeitschr. f. physiol. Chem.*, xvi, p. 321, 1892.

the amount of dextrose formed by Soxhlet's modification of Fehling's method give the following results:

TEMPERATURE 30°C.			TEMPERATURE 40.2°C.		
Time Minutes	Salicin Hydrolyzed	$K \cdot 10^5$	Time Minutes	Salicin Hydrolyzed	$K \cdot 10^5$
$t$	$z = \frac{x}{a}$	$\frac{10^5}{t} \log_{10} \frac{1}{1-z}$	$t$	$z = \frac{x}{a}$	$\frac{10^5}{t} \log_{10} \frac{1}{1-z}$
20	0.095	217	20	0.142	333
40	0.166	197	40	0.249	311
61	0.248	203	60	0.344	305
81	0.320	207	80	0.427	302
105	0.391	205	105	0.498	285
135	0.444	189	118	0.554	297
155	0.492	190	135	0.610	303
			153	0.658	305
Mean.....		201	Mean.....		305

The later investigations show a similar tendency towards constancy during the first period of the reaction. Hudson and Paine<sup>9</sup> point out that the results to which Henri<sup>10</sup> and Herzog<sup>11</sup> apply their formulæ do not take into account the mutarotation of the  $\beta$ -glucose split off from the salicin. They show, moreover, that, if this condition is corrected by the addition of a little sodium carbonate solution, the course of hydrolysis of a 5 per cent salicin solution at 30°C. measured by the change in the optical rotation gives satisfactory values for the unimolecular constant during the short time period investigated. Likewise, Auld's<sup>12</sup> experiments on the decomposition of salicin by phaseolunataase show a satisfactory constancy for  $K$  of the first order during a three-hour digestion period. For a short time period, therefore, the unimolecular formula appears to give a satisfactory expression of the time relations.

A similar lack of agreement exists with respect to the relation between the enzyme concentration and the amount of hydrolysis. The simplest condition to be expected, if the reaction follows the law of mass action, is that of direct proportionality between the

<sup>9</sup> Hudson and Paine: *Journ. Amer. Chem. Soc.*, xxxi, p. 1242, 1909.

<sup>10</sup> *Loc. cit.*

<sup>11</sup> *Loc. cit.*

<sup>12</sup> Auld: *Journ. Chem. Soc.*, xciii, p. 1251, 1903.

enzyme concentration and the velocity of hydrolysis as expressed by the formula,  $\log_{10} \frac{a}{a-x} = Kft$ , in which  $a$  represents the initial concentration of the substrate,  $x$  the amount transformed in the time  $t$  and  $K$  the velocity constant. The relation has been confirmed for a few ferments, particularly when the substrate concentration was kept constant. Thus Walters<sup>13</sup> in a very thorough investigation on the hydrolysis of casein by trypsin was able to show that this ferment under suitable experimental conditions obeys the law of direct proportionality not only with a constant substrate concentration, but also when this is varied within fairly wide limits. Calculations from Tammann's<sup>14</sup> results, based on the amount of hydrolysis of a 3 per cent salicin solution at 28°C. in twelve hours measured by Fehling's method, likewise show that there is a direct proportionality between the enzyme concentration and the velocity of hydrolysis through a wide range of enzyme concentrations. The relation is given in the following table in the column headed,  $\frac{K \cdot 10^2}{C}$ , in which ratio  $K$  represents the velocity constant and  $C$  the enzyme concentration expressed in milligrams.

EMULSIN	$z = \frac{x}{a}$		$K \cdot 10^2$	$\frac{K \cdot 10^2}{C}$
<i>mgms.</i>				
0.078	0.028	0.023	92	1.18
0.156	0.058	0.048	197	1.26
0.312	0.102	0.092	369	1.18
0.625	0.203	0.211	839	1.34
1.250	0.347	0.355	1565	1.25
2.500	0.593	0.600	3289	1.32
5.000	0.800	0.805	5870	1.17
10.000	0.913	0.913	8838	(0.88)
Mean.....				1.24

Aside from the highest enzyme concentration, the ratio shows no very marked or regular fluctuations in either direction, and is, therefore, very satisfactory.

<sup>13</sup> Walters: this *Journal*, x, p. 267, 1912.

<sup>14</sup> Tammann: *Zeitschr. f. physikal. Chem.*, xviii, p. 480, 1895.

It appears, therefore, that under proper conditions the hydrolysis of salicin by emulsin follows the unimolecular time law and the law of direct proportionality between enzyme concentration and velocity of hydrolysis. These conditions are satisfied in neutral solutions when the digestion period is short and the enzyme concentration and temperature such as to give rapid decomposition of the substrate. According to Compton<sup>15</sup> the optimum temperature for this reaction is 34°C. These conditions have been satisfied in the experiments reported herewith, by measuring the amount of hydrolysis in most cases during a two-hour digestion period at temperatures ranging from 35 to 40°C.

## II. EXPERIMENTAL.

### *a. General procedure.*

These investigations on the behavior of emulsin solutions in the presence of collodion membranes indicate that the phenomenon is one of absorption and does not differ essentially from absorption by other solid media as, for example, animal charcoal. On account of this lack of agreement with the results previously reported it has been considered important to outline the experimental conditions in some detail.

Anthony's "negative cotton," manufactured by the Ansco Company, dissolved in a mixture of equal parts of alcohol and ether to make about a 5 per cent solution was used in the preparation of the collodion films. It was found necessary to dilute this from time to time with alcohol-ether mixture to compensate for that lost by evaporation during preparation of the films. If the solution is too thick, air bubbles thrown into the solution by dipping in the glass rods or plates rise to the surface very slowly, and poorly prepared films containing many air bubbles result. Such films are uncertain in their action. The solution was, therefore, kept just thin enough to avoid this difficulty.

For much of this work, Porter's<sup>16</sup> method of using the collodion in the form of small tubes was followed. Short lengths of glass tubing of the proper bore were rounded off at one end so as to

<sup>15</sup> Compton: *Proc. Roy. Soc., B*, lxxxvii, p. 245, 1914.

<sup>16</sup> *Loc. cit.*

leave a sufficient aperture for the free passage of water. This aperture was sealed by dipping the end into the collodion solution and at the same time preventing the solution from entering the tube by closing the free end with the finger. The tubes were now prepared by dipping these glass rods to the required depth in the collodion solution until the desired thickness was obtained, allowing the films to dry a minute or two between successive dips. As much uniformity as possible was attained by making all the tubes for an experiment at the same time, and by keeping the time between each dip as well as the number of times dipped the same throughout. The films were allowed to dry for two minutes after the last dip, whereupon they were placed in distilled water until they had set. When the tubes had set sufficiently they were carefully drawn off and kept in distilled water until used. By changing the water occasionally they were freed from the last traces of alcohol and ether. The tubes thus prepared were clear, elastic, free from bubbles, and uniform. They were 5 mm. in diameter, and 1 cc. of a solution, therefore, occupied a depth of about 5 cm. and exposed a surface of about 7.85 sq. cm. to the action of the tube. The tube lengths referred to in some of the experiments were made by cutting up these tubes into pieces about 2 cm. long.

Collodion discs were made in the same way by dipping a glass plate in the solution. After the films had been prepared and allowed to set, discs 5 cm. in diameter were cut out and kept in distilled water until used.

The enzyme used was Merck's commercial emulsin preparation manufactured from sweet almonds. This preparation contains a small insoluble residue, and consequently it was necessary to use filtered solutions. Merck's salicin and amygdalin were used as substrates.

The amount of action was determined in all cases by analyzing the samples for dextrose according to Bertrand's<sup>17</sup> method. If samples are diluted so that a 20-cc. aliquot gives a titration equivalent to not more than 25 mgms. of dextrose, this method allows of accurate and at the same time rapid determinations.

The hydrolytic action of the enzyme was stopped by adding

<sup>17</sup> Bertrand: *Bull. soc. chim.*, xxv, p. 1285, 1906.

1 cc. of  $\frac{N}{1}$  sodium carbonate solution per 10 cc. of digest.<sup>18</sup> The samples were analyzed as soon as possible in order to avoid any destruction of glucose due to alkalinity of the medium.

Toluol was used as a disinfectant, although trouble was sometimes occasioned by its rapid evaporation. In experiments requiring long time intervals the toluol was renewed as often as was necessary to compensate for such loss and insure sterility in the cultures. According to the statement in Euler,<sup>19</sup> the enzyme is moderately resistant to this reagent.

*b. The time relations involved in the absorption of emulsin by collodion.*

The time relations involved in the absorption of enzymes by solid absorption media have not been accurately determined for any enzyme. In general it has been observed that the reaction proceeds rapidly until the greater part of the enzyme has been absorbed, and then goes on slowly until absorption is complete. From Hedin's<sup>20</sup> investigations in which some attention was paid to the time relations involved in the absorption of rennet by animal charcoal, the only conclusion that can be drawn is that the reaction is a rapid one. Dyer and Douglas<sup>21</sup> likewise found that the absorption of trypsin by animal charcoal was very rapid during the initial stages of the reaction. After about thirty minutes' contact the effect of the trypsin when measured on 10 per cent gelatin jelly was only slightly less than 13 per cent of the original activity. There were still traces of trypsin in the solution after a period of seven hours, but only enough to give 0.1 per cent of the original activity. The following experiments were conducted to determine the time course followed in the absorption of emulsin by collodion.

EXPERIMENT 1. Forty-eight collodion tubes were drained as free from water as possible and each placed in a 30-cc. test tube provided with a tightly fitting rubber stopper. The test tubes were then placed in the incubator, and, after they had reached the temperature of the incubator, 0.2 cc. of

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<sup>18</sup> Hudson and Paine: *loc. cit.*

<sup>19</sup> *Loc. cit.*, p. 175.

<sup>20</sup> Hedin: *Zeitschr. f. physiol. Chem.*, lx, p. 364, 1909.

<sup>21</sup> Dyer and Douglas: *Proc. Roy. Soc., B*, lxxxii, p. 168, 1910.

of the flask were rotated before the removal of each sample. A temperature of  $37.5^{\circ}\text{C.} \pm 1^{\circ}$  was maintained throughout the experiment. The results of this experiment are tabulated in Table II in which each figure represents the mean of two determinations.

The results in the above tables have been calculated in accordance with the principles already laid down. By dividing both terms of the fraction  $\frac{a}{a-x}$  in the formula  $K = \frac{1}{t} \log_{10} \frac{a}{a-x}$

by  $a$ , the equation  $K = \frac{1}{t} \log_{10} \frac{1}{1-\frac{x}{a}}$ , is obtained. Now by sub-

stituting  $z = \frac{x}{a}$  in this equation, the unimolecular formula is

obtained in the form,  $K = \frac{1}{t} \log_{10} \frac{1}{1-z}$ , which is the form that

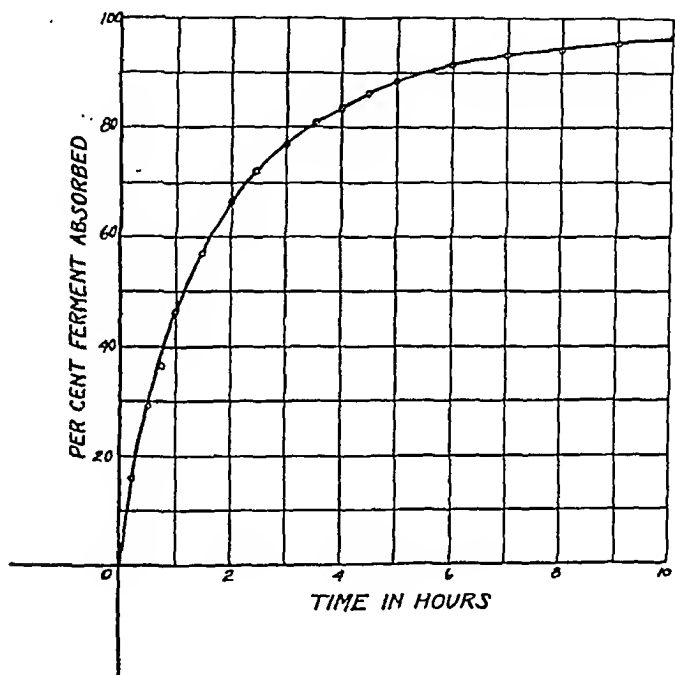
has been used in the above calculations. In the ratio,  $z = \frac{x}{a}$ ,  $x$

represents the amount of salicin decomposed during the two-hour digestion period, and  $a$  the initial salicin concentration. From these ratios the velocity constants for the enzyme solution after the number of minutes' contact with collodion noted in the table have been calculated by substitution in the above formula. Now, if the enzyme concentrations are directly proportional to the velocity constants, when other conditions are the same throughout,

then the ratio  $z = \frac{y}{c}$  in which  $y$  is the amount of activity lost

at any time under consideration, and  $c$  the velocity constant of the enzyme solution before contact with collodion, 1149 in the first experiment and 925 in the second, will give the relative enzyme concentrations absorbed after the number of minutes' contact with collodion noted in the tables. These figures can, of course, be read directly as percentages by moving the decimal point two places to the right. In spite of the different experimental conditions these figures show a very close agreement for the two experiments. The accompanying figure in which the ordinates represent the percentages of emulsin absorbed and the abscissae the time in hours represents graphically the course of absorption followed in Experiment 2.

If this absorption follows the unimolecular time law, then substitution in the formula  $K = \frac{1}{t} \log_{10} \frac{1}{1-z}$ , where  $z = \frac{y}{c}$ , should give constant values for  $K$ . These values, however, given in the last column of each table, show a marked decrease throughout the experiment. This decrease is no more marked than that which has been reported by some investigators for the time course of



the hydrolysis of salicin by emulsin. Nevertheless it is analogous to the behavior of some absorption phenomena to which it might be compared. For the absorption of congo red by filter paper, Bayliss<sup>22</sup> has reported observations from which the following calculations have been made:

<sup>22</sup> Bayliss: *Biochem. Journ.*, i, p. 175, 1906.

TIME MINUTES	CONGO RED	CONGO RED ABSORBED	$K \cdot 10^4$
	per cent	$z = \frac{x}{a}$	$\frac{10^4}{t} \log_{10} \frac{1}{1-z}$
5	98	0.036	318
30	90	0.182	291
100	70	0.545	342
200	60	0.727	282
420	55	0.818	176
840	48	0.945	150
1440	45	1.000	139

Since the equilibrium point was reached when 45 per cent of the dye was still unabsorbed, this has been regarded as 100 per cent absorption in the calculations. The same fall in the values of  $K$  may be noted. Similarly, Rakowski<sup>23</sup> calls attention to the fact that in the hydration or dehydration of starch, the first two-thirds of the water is taken up or lost during the first twenty-four hours, whereas the remaining one-third requires two months, and calculations according to the unimolecular formula give diminishing values. From these facts it is plain that the rate of absorption gives no evidence of a chemical change within the solution, but rather establishes its relation to other absorption phenomena determined by physical forces, of which diffusion is probably a determining factor in this particular instance.

*c. The temperature relations in the absorption of emulsin by collodion.*

The transformations involved in the hydrolysis of glucosides by emulsin and in the spontaneous inactivation of emulsin both in solution and in the dry state give the temperature coefficients expected for chemical reactions. Thus Tammann<sup>24</sup> found for the hydrolysis of salicin by emulsin a temperature coefficient of 2.4 for the temperature interval 15–25°C., giving for  $\mu$  the value 15,000, calculated from the temperature formula of Arrhenius. Calculations from the values given by Compton<sup>25</sup> for the hydrolysis of salicin by emulsin at 17.7° and 34.7°C. give a temper-

<sup>23</sup> Rakowski: *Journ. Russ. phys.-chem. Soc.*, xliv, p. 836, 1912.

<sup>24</sup> Tammann: *Zeitschr. f. physikal. Chem.*, xviii, p. 426, 1895.

<sup>25</sup> *Loc. cit.*

ature coefficient of 1.8 for a ten-degree interval. The temperature coefficient for the hydrolysis of amygdalin as observed by Auld<sup>26</sup> for ten-degree intervals between 15° and 50°C. varied from 1.37 to 2.37 with a mean value for six different temperature intervals of 1.85. Likewise Tammann<sup>27</sup> has shown that for the spontaneous inactivation of emulsin in 0.5 per cent solution  $\mu$  has the value 45,000, and for the spontaneous decomposition of dry emulsin 26,300.

On the other hand, the absorption of congo red by filter paper gives according to Bayliss<sup>28</sup> a temperature coefficient of 1.36 for the ten-degree interval 40° to 50°C. The following investigation was undertaken to determine the temperature coefficient for the absorption of emulsin by collodion:

EXPERIMENT 3. A series of small water baths was prepared and temperatures maintained in them at 25°, 30°, 35°, 40°, and 45°C. with a variation of about  $\pm 1^\circ$  in each case. Five 100-cc. flasks nearly filled with collodion tube lengths were drained as free from water as possible and one placed in each of the water baths. When they had reached the temperature of the water baths, 50 cc. of a 0.5 per cent solution of emulsin previously warmed to the temperature of the water bath were added to each flask. Samples were withdrawn as in Experiment 2 at the end of 30, 60 and 150 minutes and their activity determined on 10 cc. of a 5 per cent solution of salicin by determining the amount of dextrose split off in two hours at 37.5°C.  $\pm 1^\circ$ . The determination of the original activity was made from the 25° flask by taking out a sample immediately after adding the emulsin solution and quickly rotating it. It was thought that the rapid initial period of absorption would be avoided as much as possible at this temperature. Each result in Table III is the mean of two determinations.

It will be noted that the temperature series for 45°C. gives lower absorption values than that for 40°C. Whether this is due simply to some experimental condition was not investigated, but it may possibly be connected with the fact which Gaucher<sup>29</sup> has reported that the porosity of collodion filters diminishes with increasing temperature. It will be noted that the temperature coefficients are all low, the average for the series being 1.17. This is of the order of magnitude to be expected for a reaction determined by physical forces.

<sup>26</sup> *Loc. cit.*

<sup>27</sup> *Loc. cit.*

<sup>28</sup> *Loc. cit.*

<sup>29</sup> Gaucher: *Bull. des sciences pharm.*, xix, p. 129, 1913.

TABLE III.

*Series A. Temperature 25°C.*

TIME MINUTES	DEXTROSE FORMED	SALICIN HYDROLYZED	$K \cdot 10^4$	EMULSIN ABSORBED	TEMPERATURE COEFFICIENT
	<i>mgms.</i>	$z = \frac{x}{a}$	$\frac{10^4}{2} \log_{10} \frac{1}{1-z}$	$z = \frac{y}{c}$	$\frac{K_t + 10^4}{K_t^0}$
0	106.6	0.337	892		
30	81.8	0.258	648	0.274	
60	70.5	0.223	548	0.386	
150	44.2	0.140	328	0.632	

*Series B. Temperature 30°C.*

0	106.6	0.337	892		
30	76.5	0.242	602	0.325	
60	62.9	0.199	482	0.460	
150	37.1	0.117	270	0.697	

*Series C. Temperature 35°C.*

0	106.6	0.337	892		
30	74.7	0.236	585	0.344	1.26
60	59.4	0.188	452	0.493	1.28
150	35.0	0.111	256	0.713	1.13
Mean .....					1.22

*Series D. Temperature 40°C.*

0	106.6	0.337	892		
30	68.7	0.217	531	0.405	1.25
60	50.9	0.161	381	0.573	1.25
150	26.5	0.084	191	0.786	1.13
Mean .....					1.21

*Series E. Temperature 45°C.*

0	106.6	0.337	892		
30	72.0	0.228	562	0.370	1.08
60	56.0	0.177	423	0.526	1.07
150	30.1	0.095	217	0.757	1.06
Mean .....					1.07

*d. Inhibition in inactivated emulsin solutions.*

The development of inhibitive substances by enzyme solutions in various forms of treatment has been observed for many enzymes, although the exact nature of these bodies has not been satisfactorily fixed in most cases. Some inhibitive substances, however, have been considered as anti-enzymes, which were able not only to antagonize the enzyme for which they were specifically inhibitive, but were able also to effect the reverse synthesis. Thus Beitzke and Neuberg<sup>30</sup> reported that the serum obtained from rabbits after repeated intraperitoneal injection of emulsin solutions was able to effect the synthesis of lactose from galactose and glucose. Bayliss,<sup>31</sup> however, has shown that the inhibitive effect of such serum is due to the diminution of the optimal acidity, and that the same effect is produced by normal and by immune serum. Neither serum, however, was found to have any synthetic action on a mixture of lactose and galactose. Porter's<sup>32</sup> observation that enzyme solutions inactivated by contact with collodion membranes possessed inhibitory powers which were only in part explained by the inhibitory action of substances preformed in the solutions led to the conclusion that the enzymes themselves were changed into inhibitive substances of a zymoid, or possibly in part true anti-ferment, character. For emulsin an average inhibition amounting to 24 per cent was observed when the activity was measured on salicin.

I have repeatedly attempted to confirm these results but without success. A slight acceleration in activity was occasionally noted, but never any significant inhibition. In an experiment in which the activity of 0.1 per cent emulsin solutions was measured by three hour's digestion of 10 cc. of 2 per cent amygdalin solution at 40°, the following results were secured:

<sup>30</sup> Beitzke and Neuberg: *Virchow's Archiv*, clxxxiii, p. 169, 1906.

<sup>31</sup> Bayliss: *Journ. of Physiol.*, xliii, p. 455, 1912.

<sup>32</sup> *Loc. cit.*

10 CC. AMYODALIN SOLUTION +	DEXTROSE
	<i>mgms.</i>
9 cc. water + 1 cc. untreated emulsin .....	67.9
9 cc. treated emulsin + 1 cc. untreated emulsin .....	74.1
9 cc. treated emulsin (boiled) + 1 cc. untreated emulsin.	53.2
10 cc. treated emulsin.....	5.8

These results were further confirmed by the following more extensive experiment:

EXPERIMENT 4. A 100-cc. flask nearly filled with collodion tube lengths was drained as free from water as possible. It was brought to the temperature of the incubator, and then 50 cc. of a 1 per cent solution of emulsin was added to it. At the expiration of the intervals noted in the table, 0.5-cc. samples of this treated emulsin solution were withdrawn and their action tested with an untreated emulsin solution by the digesting power on 10 cc. of a 5 per cent salicin solution for two hours. Three duplicate observations were made in each case as follows: (1) 0.5 cc. of the treated emulsin solution alone to give the activity of the treated preparation, (2) 0.5 cc. of the untreated emulsin solution to give the activity of the untreated solution, and (3) 0.5-cc. portions of treated emulsin solutions were added to each of two test tubes containing salicin solution. These tubes were then shaken to distribute the contents uniformly, and then after five minutes 0.5 cc. of untreated emulsin solution was added to each test tube and the contents thoroughly mixed and digestion allowed to proceed for two hours. This gave the activity of the mixed solutions. After 308 hours the experiment was varied as noted in the separate table compiled from these results. In order to determine whether the collodion tubes themselves held any considerable amount of enzyme the action of ten of the tube lengths was tested on salicin solution. A temperature of  $35^{\circ}\text{C.} \pm 1^{\circ}$  was maintained throughout this experiment.

TABLE IV.

CONTACT WITH COLLODION	SALICIN HYDROLYZED		
	Untreated	Mixed	Treated
<i>hours</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
20	28.2	28.9	1.3
46	28.6	28.9	0.6
64	23.6	25.9	0.5
92	25.4	26.5	
160	19.7	22.0	
218	18.1	22.4	

The following results were secured after 308 hours:

TABLE V.

10 cc. SALICIN SOLUTION +	SALICIN HYDROLYZED
	<i>per cent</i>
1 cc. untreated + 1 cc. treated + 10 cc. water.....	39.1
1 cc. untreated + 10 cc. treated + 1 cc. water.....	45.2
1 cc. untreated + 11 cc. water.....	34.8
10 tube lengths + 10 cc. water.....	41.9
1 cc. treated + 11 cc. water.....	0.4

These results show that any inhibition must have been due to substances preformed in the solutions. The presence of such inhibitive substances in stomach extracts has been observed by Weinland<sup>33</sup> and Porter<sup>34</sup> calls attention to the fact that the stomach extracts she used contained large quantities of antipepsin, although she still obtained inhibition with purified preparations. That such substances probably occur in emulsin solutions has been suggested by Cramer and Bearn<sup>35</sup> who found a considerable inhibition when solutions heated to 60° and to 100° were tested against fresh emulsin solutions. They suggest that these inhibitory substances only become evident when the hydrolytic power of the enzyme is destroyed by heat. The results given above, however, show that an enzyme solution which had lost practically all its enzyme content by absorption developed a considerable inhibitory power on heating; and therefore heating may have an effect in producing these bodies as well as in unmasking them, and perhaps from non-ferment substances in the solution. The commercial preparation of emulsin contains a considerable amount of protein substances, whereas Bayliss<sup>36</sup> has shown that the ferment that hydrolyzes  $\beta$ -glucosides is not a protein, and Ohta<sup>37</sup> has prepared a protein-free emulsin.

The development of such inhibitive powers, then, in an inactivated solution would depend on differential absorption, the enzyme being absorbed and the inhibitive substance remaining in

<sup>33</sup> Weinland: *Zeitschr. f. Biol.*, xlv, p. 45, 1903.

<sup>34</sup> *Loc. cit.*

<sup>35</sup> Cramer and Bearn: *Biochem. Journ.*, ii, p. 174, 1907.

<sup>36</sup> Bayliss: *Journ. of Physiol.*, xliii, p. 455, 1912.

<sup>37</sup> Ohta: *Biochem. Zeitschr.*, lviii, p. 329, 1913.

solution. Porter<sup>38</sup> has shown that this is possible. In the dialysis of emulsin through collodion films, it was found that the emulsin became inactive and that both dialysate and diffusate exhibited inhibitive properties. Eriksson<sup>39</sup> similarly has demonstrated the presence of an inhibitory substance in invertase solutions, which is not absorbed to any great extent by animal charcoal, although the absorption of invertase was very marked. These results show, therefore, that there is no change of emulsin into an inhibitive substance which is set free in the inactivated solution, but that such action must be ascribed to differential absorption.

*e. The recovery of emulsin absorbed by collodion.*

In a preliminary experiment in which the behavior of emulsin solutions in contact with collodion tubes was under investigation, a 0.1 per cent solution of emulsin which showed an activity when measured on 10 cc. of a 2 per cent amygdalin solution such as to give a titration of 10.4 cc. permanganate solution, showed after 5.5 hours' contact with collodion a reduction in activity to 1.75. By mistake the collodion tube was left submerged in one of the three samples and it showed an activity of 5.7. These data are collected in the following table.

TABLE VI.

CONTACT WITH COLLODION	DEXTROSE FORMED	AMYGDALIN HYDROLYZED	$K \cdot 10^3$	RELATIVE ACTIVITY
hours	mgms.	$z = \frac{x}{a}$	$\frac{10^3}{2} \log_{10} \frac{1}{1-z}$	per cent
0	53.6	0.381	104	100.0
5.5	8.4	0.060	13	12.5
Tube	28.2	0.200	48	46.3

From these results it appears that about 33.8 per cent of the emulsin originally present in the solution was absorbed by the collodion tubes and was still capable of extraction by an amygdalin solution after five and one-half hours. Since 87.5 per cent of the emulsin had been absorbed by the tubes, it would appear that about 40 per cent of the enzyme absorbed was still unchanged. Similarly the tube lengths in Experiment 4 showed a considerable

<sup>38</sup> *Loc. cit.*

<sup>39</sup> Eriksson: *Zeitschr. f. physiol. Chem.*, lxiii, p. 313, 1911.

amount of active enzyme present in an absorbed but unaltered condition, but there no comparison could very well be applied as to the amount absorbed, but still unchanged, and that which had become inactive. The following more extensive experiment was performed to secure further data on this point:

EXPERIMENT 5. A series of test tubes each containing a drained collodion tube and 0.1 cc. of toluol was prepared and brought to the temperature of the incubator. To each was then added 1 cc. of a 1 per cent solution of emulsin and the tubes replaced in the incubator. After the times noted in the table, the action of the collodion tubes was determined by adding 10 cc. of a 5 per cent salicin solution to the test tubes, the ends of the collodion tubes being snipped off with a pair of scissors to allow free circulation through them. Digestion periods of one, two, and three hours were used in measuring the activity of the absorbed enzyme, and a three-hour period in measuring that of the inactivated enzyme solution. This latter was negligible in the 30-hour period and thereafter. For the 5-hour period it amounted to 7 per cent of that shown by the original enzyme solution. In order to prevent too rapid evaporation of toluol during such long periods, the rubber stoppered test tubes were further sealed by applying melted paraffin with a brush around the rim. Even then it was necessary to renew the toluol occasionally during the longer periods. A temperature of  $35^{\circ}\text{C.} \pm 1^{\circ}$  was maintained throughout the experiment. The results are tabulated in Table VII.

TABLE VII.

*Series A. Original enzyme solution.*

DIGESTION PERIOD	DEXTROSE FORMED	SALICIN HYDROLYZED	$K \cdot 10^3$	RELATIVE ACTIVITY
hours	mgms.	$z = \frac{x}{a}$	$\frac{10^3}{t} \log_{10} \frac{1}{1-z}$	per cent
1	75.6	0.239	119	
2	141.8	0.448	129	
3	188.5	0.596	131	
Mean .....			126	100

*Series B. Original enzyme solution with a collodion tube in the digesting mixture.*

1	77.4	0.245	122	
2	138.6	0.438	125	
3	185.4*	0.586	128	
Mean .....			125	99

\*This number is based on one determination.

TABLE VII.—Continued.

*Series C. Contact with collodion 5 hours.*

DIGESTION PERIOD	DEXTROSE FORMED	SALICIN HYDROLYZED	$K \cdot 10^3$	RELATIVE ACTIVITY
hours	mgms.	$z = \frac{x}{n}$	$\frac{10^3}{t} \log_{10} \frac{1}{1-z}$	per cent
1	57.0	0.180	86	
2	102.9*	0.325	85	
3	132.2	0.418	78	
Mean.....			83	66

*Series D. Contact with collodion 50 hours.*

1	44.5	0.141	66	
2	76.9	0.243	60	
3	106.9	0.338	60	
Mean.....			62	49

*Series E. Contact with collodion 60 hours.*

1	41.2	0.130	60	
2	74.7	0.236	58	
3	102.9	0.325	57	
Mean.....			58	46

*Series F. Contact with collodion 250 hours.*

1	27.9	0.088	40	
2	51.9	0.164	39	
3	72.9	0.230	38	
Mean.....			39	31

*Series G. Contact with collodion 480 hours.*

1	25.4	0.080	36	
2	50.2	0.159	38	
3	64.0	0.202	33	
Mean.....			36	29

These results show that even after twenty days, the activity of the enzyme absorbed by the collodion still amounted to 30 per cent of the original activity. This is precisely analogous to the results obtained by Hedin<sup>40</sup> who found that the action of animal charcoal on trypsin took place in two stages; first a rapid absorption of the enzyme by the charcoal, and second, a slow irreversible fixation of the enzyme. There can be no doubt from this experiment that the action of collodion on emulsin is one in which the enzyme is absorbed and retained by the collodion, and not changed into an inhibitive substance which exhibits its action in the inactivated solution.

*f. The limit to the amount of emulsin which may be absorbed by collodion.*

Hedin<sup>41</sup> found that, if a very small quantity of animal charcoal was used, it was possible to saturate the charcoal with enzyme so that any further addition of enzyme solution would not be affected. Schmidt<sup>42</sup> has likewise shown for absorption phenomena in general that the quantity increases to a maximum independent of the concentration of the surrounding medium. On the other hand, Porter<sup>43</sup> states that the inactivating power of collodion tubes appears to increase on repeated use. Obviously with the low concentrations used in enzyme work, it would be expected that the collodion is able to inactivate a large quantity of enzyme solution, especially when expressed in the hydrolyzing power of the solution inactivated. The following experiment, however, shows that the collodion does tend to become saturated as would be expected from the previous experiments.

EXPERIMENT 6. To a rather thick collodion disc 5 cm. in diameter in a 150-cc. Erlenmeyer flask were added 25 cc. of a 1 per cent emulsin solution. The activity of the emulsin was determined immediately after addition to the flask, again after five hours' contact with the collodion, fifteen hours' contact, and twenty-four hours' contact. This portion was then poured off and a fresh 25 cc. added and the same procedure followed in testing the loss of activity of this portion. The third solution was added 120 hours after

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<sup>40</sup> Hedin: *Biochem. Journ.*, i, p. 484, 1906.

<sup>41</sup> *Loc. cit.*

<sup>42</sup> Schmidt: *Zeitschr. f. physikal. Chem.*, lxxiv, p. 689, 1910.

<sup>43</sup> *Loc. cit.*

the second, the fourth, seventy-two hours after the third, and the fifth fifty-six hours after the fourth. The previous portion was always poured off immediately before adding another portion and the loss of activity measured after the number of hours' contact indicated in the table. The temperature of the incubator was  $34^{\circ}\text{C.} \pm 1^{\circ}$ , and the activity was determined as in former experiments by two hours' digestion of 10 cc. of 5 per cent salicin solution and 1 cc. of ferment solution. These results are tabulated in Table VIII in which each number represents the mean of two determinations.

TABLE VIII.

*Series A. First 25 cc. of enzyme solution.*

TIME	DEXTROSE FORMED	SALICIN HYDROLYZED	$K \cdot 10^3$	EMULSIN ABSORBED
hours	mgms.	$z = \frac{x}{a}$	$\frac{10^3}{t} \log_{10} \frac{1}{1-z}$	per cent
0	146.7	0.464	135	
5	107.5	0.340	90	33.3
15	62.2	0.197	48	64.4
24	39.1	0.124	29	78.5

*Series B. Second 25 cc. of enzyme solution.*

0	153.0	0.483	143	
5	117.1	0.370	100	30.1
15	71.1	0.224	55	61.5
120	3.5	0.011	2	98.6

*Series C. Third 25 cc. of enzyme solution.*

0	96.2	0.304	79	
5	89.6	0.283	72	8.9
15	116.5 <sup>a</sup>	0.368	50	36.7
72	39.8	0.126	29	63.3

*Series D. Fourth 25 cc. of enzyme solution.*

0	152.5	0.482	143	
5	146.6	0.463	135	5.6
15	131.1	0.414	116	18.9
56	71.0	0.224	55	61.5

*Series E. Fifth 25 cc. of enzyme solution.*

0	101.1	0.319	83	
5	98.7	0.312	81	2.4
17	84.8	0.268	68	18.1

<sup>a</sup> This number is based on a four-hour digestion period.

*g. The extraction by salicin of emulsin absorbed by collodion.*

Since in a previous experiment it appeared that the presence of a collodion tube in a digesting solution of salicin and emulsin had no effect on the course of digestion, it appeared probable that the enzyme after its absorption was able to act on salicin, and therefore that the activity of collodion tubes in a solution of salicin was not due entirely to the extraction of the enzyme by the substrate, but represented in part the activity of the enzyme still held in an absorbed condition by the collodion. This fact we have established by the following experiment:

EXPERIMENT 7. The last portion of enzyme solution was poured off from the collodion disc used in Experiment 6, the disc washed thoroughly with distilled water, and 100 cc. of a 5 per cent solution of salicin added to it. At the end of one hour this portion of salicin solution was poured off into another flask, the disc again thoroughly washed with distilled water, and a fresh 100 cc. of salicin solution added to it. The amount of hydrolysis in the first 100-cc. portion was determined on a 10-cc. sample immediately after pouring it off the disc, and thereafter at the time intervals noted in the table. The second portion of salicin solution was likewise poured off at the end of one hour's contact with the collodion disc and treated exactly like the first. The disc was again washed and the final portion of 30 cc. of fresh salicin solution added to it. A temperature of  $34^{\circ}\text{C} \pm 1^{\circ}$  was maintained throughout this experiment. The results are tabulated in Table IX in which each figure represents the mean of two determinations.

TABLE IX.  
*First 100 cc. of salicin solution.*

TIME	DEXTROSE FORMED	SALICIN HYDROLYZED	$K \cdot 10^4$
hours	mgms.	$z = \frac{x}{a}$	$\frac{10^4}{t} \log_{10} \frac{1}{1-z}$
1	400	0.126	585
2	448	0.142	333
3	479	0.151	237
80	1534	0.485	36

*Second 100 cc. of salicin solution.*

1	418	0.132	615
2	435	0.137	320
3	442	0.140	218
80	749	0.237	15

*Third 30 cc. of salicin solution.*

80	908	0.956	170
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The third addition does not give a result comparable with those given by the first and second portions of the salicin solution on account of the fact that the hydrolysis was nearly complete in this sample. It is plain, however, that the disc still retained by far the greater part of the ferment. There is a continued hydrolytic action after removal due to ferment actually extracted from the collodion, but this could not have accounted for more than 10 per cent of the action in the first case, or more than half as much in the second. Attention should, therefore, be called to the fact that the figures given for the recovery of activity are low on account of the part which diffusion must have played in bringing the salicin within the sphere of influence of the absorbed ferment.

Hedin<sup>44</sup> has reported, that in the case of trypsin absorbed by animal charcoal, the casein actually extracts the enzyme from the charcoal, and in this respect the absorption of emulsin by collodion is different. It is more nearly analogous to the behavior of invertase absorbed by colloidal ferric hydroxide and alumina as reported by Michaelis<sup>45</sup> who found that the ferment thus absorbed was still able to exert its hydrolytic activity. These results are not reported in such a manner, however, as to show conclusively whether the invertase exerted its action while still absorbed or whether it was extracted from the absorbing material. Other phenomena have, however, been reported in which the ferment was able to exert its hydrolytic action when in a precipitated form of some kind. Thus Bayliss<sup>46</sup> reports that toluidin-blue gives a precipitate with trypsin which exhibits considerable tryptic activity, and Holzberg<sup>47</sup> has investigated a similar insoluble safranin-trypsin compound. Similarly, it has been observed that emulsin is able to exert its action on gentiopierin and salicin in alcohol solution so strong that the emulsin is precipitated.<sup>48</sup> On the other hand, some absorbents yield up their absorbed enzyme very readily upon proper treatment. Michaelis and Rona<sup>49</sup> have found that rennin absorbed by mastic

<sup>44</sup> Hedin: *Biochem. Journ.*, i, p. 484, 1906; ii, p. 81, 1907.

<sup>45</sup> Michaelis: *Biochem. Zeitschr.*, vii, p. 488, 1907.

<sup>46</sup> Bayliss: *Biochem. Journ.*, i, p. 175, 1906.

<sup>47</sup> Holzberg: *this Journal*, xiv, p. 335, 1913.

<sup>48</sup> Cf. Bourquelot and Bridel: *Journ. pharm. chim.*, iv (vii), p. 385, 1911; v (vii), p. 1053, 1912; *Compt. rend.*, cliv, p. 944, 1912.

<sup>49</sup> Michaelis and Rona: *Biochem. Zeitschr.*, iv, p. 11, 1907.

still exhibits its coagulating power and is transferred to fibrin in neutral solutions, and slightly to water. By treatment with ether and then alcohol, a precipitate containing less mastic was obtained, and it readily yielded up rennin on treatment with water. Trypsin on the other hand could be completely freed from such a combination on treatment with alcohol-ether. The absorption of emulsin by collodion is, therefore, not essentially different from the relations exhibited by other absorption phenomena.

*h. The separation of absorbed ferment from collodion.*

Attention has been called to the fact that previous investigators isolated the absorbed ferment by dissolving the absorbent in some medium in which the ferment is insoluble. The following experiment was made with this end in mind:

EXPERIMENT 8. To 200-cc. Erlenmeyer flasks each containing a collodion disc were added 25-cc. portions of a 5 per cent solution of emulsin, and the flasks left to stand at room temperature with occasional rotation to facilitate absorption of the ferment. After one day's contact, one of these discs was thoroughly washed with water and then treated with alcohol-ether. A finely divided cloudy suspension resulted which ran through hardened filter paper and did not separate on centrifuging. This was found to be the result whenever the collodion discs were treated directly with alcohol-ether. No further observations were made with this material. After three days' contact, a collodion disc first placed in alcohol and then in alcohol-ether turned first opaque and then gradually crumbled up on slightly shaking giving a rather copious white, flocculent precipitate. After standing for twenty-four hours this was washed three times by centrifugalization with alcohol-ether, collected on a hardened filter paper and dried over sulphuric acid. To it was then added 20 cc. of water, and the precipitate thoroughly macerated and allowed to stand with the addition of toluol for twenty-four hours at 37.5°C. with occasional stirring. The precipitate did not dissolve noticeably, but the results will show that emulsin was present in the solution. The activity was measured as in previous cases by two hours' action on 10 cc. of 5 per cent salicin solution at 37.5°C.

TABLE X.

PREPARATION	DEXTROSE FORMED	SALICIN HYDROLYZED
	mgms.	per cent
1 cc. clear filtrate.....	37.6	11.9
1 cc. suspension.....	49.7	15.7
1 cc. 1 per cent untreated emulsin.....	165.5	52.3
1 cc. untreated + 1 cc. clear filtrate.....	193.8	61.2
1 cc. untreated + 1 cc. suspension.....	195.3	61.7

During the time of contact with the collodion the original enzyme solution had lost 20 per cent of its activity as shown by the following figures:

CONTACT WITH COLLODION	DEXTROSE FORMED	SALICIN HYDROLYZED	$K \cdot 10^3$	EMULSIN ABSORBED
<i>hours</i>	<i>mgms.</i>	$z = \frac{x}{a}$	$\frac{10^3}{2} \log_{10} \frac{1}{1-z}$	<i>per cent</i>
0	173.5	0.548	172	
75	149.5	0.472	139	19.2

These last results were obtained by testing 1-cc. portions of the original enzyme solutions diluted to five volumes with water to reduce their activity so that they might be compared with those given by the recovered enzyme. They show that somewhat less than 20 per cent of the enzyme absorbed was recovered. It is to be noted that the preparations obtained in this way have no inhibitive action against fresh emulsin solutions. This preliminary experiment, therefore, demonstrated that it is possible to separate the ferment in part from the collodion.

### III. DISCUSSION AND RESUMÉ OF RESULTS.

Michaelis and Ehrenreich<sup>50</sup> have shown that in some cases the absorption of ferments depends on the electro-chemical properties of the absorbing medium and of the ferments absorbed. Thus invertase is absorbed by electro-positive substances, colloidal ferric hydroxide and alumina, and not by electro-negative substances, kaolin and colloidal arsenic sulphide. It is therefore electro-negative or acid in its behavior. These facts Michaelis<sup>51</sup> confirmed by the analogous behavior of acid and basic dyes to these absorbing agents. Rosenthaler<sup>52</sup> by the same method has shown that emulsin has the character of an acid substance. Collodion, however, as Porter<sup>53</sup> has shown absorbs a large number of ferments some of which are acid in character and some of which are basic. Moreover collodion discs absorb acid dyes such as eosin, and basic

<sup>50</sup> Michaelis and Ehrenreich: *Biochem. Zeitschr.*, x, p. 283, 1903.

<sup>51</sup> Michaelis: *ibid*, vii, p. 488, 1907.

<sup>52</sup> Rosenthaler: *ibid*, xxvi, p. 1, 1910; cf. also, Michaelis and Davidsohn: *ibid*, xxxv, p. 386, 1911.

<sup>53</sup> *Loc. cit.*

dyes, methyl violet, methylene-blue, and fuchsin from their solutions, and the basic somewhat more decidedly than the acid. As might be expected, therefore, the absorption of emulsin by collodion cannot be explained on the basis of the æcidic or basic character of the reacting substances. In this respect collodion behaves more nearly like charcoal which Michaelis<sup>54</sup> found was not selective in its behavior either toward dyes or ferments. An examination of the residue obtained by dissolving collodion from treated discs, however, showed that proteins as well as emulsin were absorbed. What effect removal of these might have on absorption has not been studied, but obviously may be of some importance in connection with the true nature of the process under investigation.

The process must be determined by physical forces, of which diffusion very likely distorts the relations here obtained. It has been stated by Porter<sup>55</sup> and confirmed by me that poorly made collodion films or those made by pouring collodion solution on a water surface are quite ineffective in absorbing enzymes from their solutions. The difference between such films and those more carefully prepared is in large part at least a difference in porosity. The immediate process of absorption, therefore, although affected by diffusion may be determined by capillary forces. Now Cameron and Bell<sup>56</sup> and later Ostwald<sup>57</sup> and Goppelroeder<sup>58</sup> have shown that the time relations involved in capillary phenomena such as the absorption of water by a column of sand or by a strip of filter paper may be expressed by the formula  $x = Kt^m$ , when  $x$  represents the amount absorbed in the time  $t$ , and  $K$  and  $m$  are constants for the reaction. For the proteins this relation has been confirmed by Robertson<sup>59</sup> for the rate of solution of casein and the extraction of salmine from desiccated tissues, processes in which the rate is dependent upon the velocity with which the dissolved protein

<sup>54</sup> *Loc. cit.*

<sup>55</sup> *Loc. cit.*

<sup>56</sup> Cameron and Bell: Bull. No. 30, p. 50, Bureau of Soils, U. S. D. A., 1905; *Journ. of Physical Chem.*, x, p. 658, 1906.

<sup>57</sup> Ostwald: *Zeitschr. f. Kolloidchemie* (2 Supplement Heft), 1908.

<sup>58</sup> Goppelroeder: *Verhandl. naturforsch. Gesellsch. zu Basel*, xix, Heft 2, 1907.

<sup>59</sup> Robertson: *Journ. of Physical Chem.*, xiv, p. 377, 1910; this *Journal*, xiv, p. 237, 1913; *Arch. f. d. ges. Physiol.*, clii, p. 254, 1913.

passes out from within the protein particles. Now in these experiments, which are concerned with the rate of solution or extraction of the protein in question by the solvent, the part played by diffusion was minimized by constantly stirring the mixture, a procedure which is impossible with enzyme solutions on account of the direct inactivating effect which such treatment would have. Although the formula is not applicable to the results which have been obtained with emulsin and collodion, the rate still presents the same initial period of rapid absorption followed by a long period of slow absorption. Moreover, as Robertson<sup>60</sup> points out, processes regulated by capillarity do not present a true equilibrium, which is in harmony with the observations here presented. It appears, therefore, that this process may be the converse of the extraction of protein and solution of casein, the enzyme here being extracted from the solution whereas in the cases mentioned the protein was extracted by the solution from its solid menstrum.

The subsequent fixation of the enzyme is a relatively slow process, probably slower than the experiments indicate on account of the fact that the active ferment is not actually extracted to any extent from the collodion. It has often been observed that enzyme solutions undergo a slow spontaneous inactivation, but this is too slow to account for the fixation by collodion. It may be possible that this fixation is of the nature of a condensation phenomenon similar to those exhibited by proteins.<sup>61</sup>

#### SUMMARY.

1. The time course of the absorption of emulsin by collodion films gives decreasing values for the unimolecular constant, and gives a curve similar to that for processes determined by capillary forces.

2. Determinations of the temperature coefficients between 25° and 45° give an average value of 1.17 for a ten-degree interval.

3. There is no evidence of the conversion of enzyme into inhibitive substances.

<sup>60</sup> *Loc. cit.*

<sup>61</sup> Cf. Robertson: *Die physikalische Chemie der Proteine*, Dresden. Theodor Steinkopff, p. 306, 1912.

4. It is possible to regain a large part of the absorbed enzyme when the collodion films are placed in contact with solutions of the substrate.

5. The activity shown by collodion films in a solution of the substrate is dependent for the most part on active enzyme still held absorbed by the collodion.

6. The absorptive power of collodion films decreases with repeated use.

7. It is possible to recover part of the enzyme from the collodion by properly dissolving the collodion with alcohol and ether.

8. It is suggested that capillary forces may account for the absorption of emulsin by collodion, and that fixation may be a surface condensation phenomenon.

It is a pleasure to thank Dr. Robertson for his *keen interest and* advice in this work, and the other members of the Physiology Staff for the assistance which they have kindly given.



# THE ACTION OF LEUCOCYTES AND KIDNEY TISSUE ON PYRUVIC ACID.

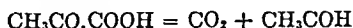
BY P. A. LEVENE AND G. M. MEYER.

(From the Laboratories of the Rockefeller Institute for Medical Research,  
New York.)

(Received for publication, March 13, 1914.)

The knowledge of the mechanism of "glycolysis" in animal tissues remains very meager. Definitely established is the formation of lactic acid in one of the intermediate phases of the process. It is probable that the latter phase is preceded by the appearance of pyruvic aldehyde. The phases which follow the formation of lactic acid and which lead to the production of carbon dioxide and of water are not known.

The mechanism of sugar metamorphosis in plants, and particularly in yeast, has been studied more successfully. Recently Neuberg<sup>1</sup> and his collaborators have brought forward a number of observations which indicate that in the process of alcoholic fermentation pyruvic acid is formed as an intermediate stage. This substance then dissociates into carbon dioxide and acetaldehyde, according to the following reaction.



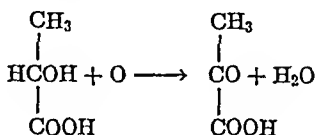
It seemed possible that also in the animal organism the conversion of hexose into carbon dioxide and water is preceded by the formation of pyruvic acid. Tschernorutzky,<sup>2</sup> working in Neuberg's laboratory, offered evidence in support of this hypothesis. This worker has claimed that animal tissues possess the power of cleaving pyruvic acid in a manner leading to the formation of carbon dioxide. However, Tschernorutzky failed to furnish satisfactory evidence that bacteria did not play the part ascribed by him to the tissues. Animal experiments with feeding pyruvic acid were performed by

<sup>1</sup> *Biochem. Zeitschr.*, xxxvii, p. 170, 1911; *ibid.*, liii, p. 406, 1913.

<sup>2</sup> *Ibid.*, xliii, p. 486, 1912.

Paul Mayer, Dakin, Ringer and Embden<sup>3</sup> and on the basis of their work one feels justified in assuming that pyruvic acid may be formed in course of the many transformations which dextrose suffers in the organism.

In our previous experiments with aseptic kidney tissue, or with leucocytes we were unable to bring about an oxidation of lactic acid. Since *a priori* it is possible that two specific enzymes are required for the final oxidation of lactic acid into carbon dioxide and water: namely, one transforming lactic acid to pyruvic acid according to the following reaction:



and the second leading to formation of carbon dioxide, it seemed possible that the kidney tissue and the leucocytes did not possess the first enzyme and yet had the second. For this reason we concluded to repeat the experiments of Tschernorutzky under absolutely aseptic conditions. The experiments were not performed with liver tissue for the reason that it is practically impossible to obtain liver tissue free from bacteria.

Very recently we noted that Ida Smedley MacLean<sup>4</sup> had also been engaged in work on the same problem but has temporarily abandoned the investigation for the lack of an adequate analytical method.

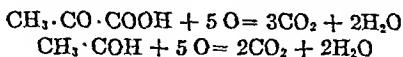
The conclusions regarding the fate of pyruvic acid were based first on the production or non-production of carbon dioxide in the course of the reaction, and second on the behavior of the reaction mixtures towards potassium permanganate.

It was reasoned that if the decomposition of pyruvic acid in animal tissues proceeds in the same manner as in the yeast, the reaction remaining at the acetaldehyde phase, then the oxygen requirement for final transformation of the products into carbon

<sup>3</sup> Mayer: *Biochem. Zeitschr.*, xlix, p. 486, 1913; Dakin and Janney: *this Journal*, xv, p. 177, 1913; Ringer: *ibid.*, xv, p. 145, 1913; Embden and Oppenheimer: *Biochem. Zeitschr.*, lv, p. 335, 1913.

<sup>4</sup> *Biochem. Journ.*, vii, p. 611, 1913.

dioxide and water remains the same as for the conversion of pyruvic acid. This is made evident from the following:



For either one of the two reactions five atoms of oxygen are required.

If, however, pyruvic acid, or the products of dissociation suffered oxidation, or if the acetaldehyde underwent reduction, then the oxygen requirement for final conversion of the products into carbonic acid and water would differ from that for pyruvic acid.

It was found that the yeast enzyme failed to act on pyruvic acid at 37°C., but at 42° caused intense evolution of carbon dioxide as stated by Neuberg and coworkers. Hence our experiments were conducted at temperatures varying between 37° and 42°C.

However, in all the experiments, provided they remained perfectly aseptic, there was never observed either the formation of carbon dioxide or a change of the oxygen requirement for transformation of the reaction product into carbon dioxide and water. Hence there is no reason to assume that under conditions of perfect asepsis leucocytes or kidney tissue are capable of decomposing pyruvic acid.

It may be mentioned here that the presence of pyruvic acid does not in any way affect the accurate estimation of carbon dioxide by means of distillation under diminished pressure. Also by means of a barium hydroxide solution it is possible to remove accurately all the carbon dioxide in the presence of pyruvic acid if the operation is performed at low temperature. However, it was not found possible to apply the method in the present work for the reason that in the presence of the autolytic products of the tissues the barium carbonate precipitate was quite flocculent and mechanically carried with it small quantities of pyruvic acid.

The bacteriological part of this work was done by Dr. Martha Wollstein, to whom we wish to acknowledge our indebtedness.

It may be also mentioned here that the evolution of carbonic acid gas was only observed in some and not in all experiments contaminated with bacterial growth.

## EXPERIMENTAL.

*Tissues.* Leucocytes were obtained from dogs by the injection of turpentine into the pleural cavity by the method described in a previous communication. Kidneys from dogs and rabbits were used. They were removed aseptically from exsanguinated animals.

*Solutions.* Pyruvic acid was prepared according to the method of Simon,<sup>5</sup> and refractionated several times. It boiled constant at 68°, under 11 mm. pressure. In order to obtain all solutions sterile without decomposing the pyruvic acid it was necessary to sterilize the pyruvic acid, sodium hydrate and Henderson phosphate solutions separately and then mix them immediately prior to adding the leucocytes or the minced kidneys. The substances were always used in the following proportions: Pyruvic acid, 1.0 gram, Henderson phosphate solution, 100 cc. and 9 cc. of a 5 per cent sodium hydroxide solution. The sodium hydroxide was prepared very carefully and was freed from carbonates.<sup>6</sup> Inasmuch as the controls had to be brought to a boil to destroy any enzymes and as this would also decompose the pyruvic acid, a somewhat different procedure was necessary. The kidney pulp was added to the phosphate solution, this brought to a boil, and when cold the pyruvic acid was added which had previously been neutralized with sodium hydroxide. The tissue mixture was allowed to stand at 37°, 40° and 42° for seven days.

*Bacteriological controls.* Smears and cultures were made of all tissue mixtures prior to analysis and only those free from all contamination were considered.

**METHODS OF ANALYSIS.** *Oxidation with permanganate.* The total oxidation as described by Greifenhagen, König and Scholl<sup>7</sup> and also used by us in a previous investigation<sup>8</sup> was used and found to give theoretical values with pyruvic acid. The tissue mixtures, after straining through cheese cloth, were freed from protein with metaphosphoric acid and the filtrate made up to 500 cc. Of this 20 cc. were used for each oxidation.

*Carbon dioxide determination.* It was found that in the cold

<sup>5</sup> Bull. soc. chim., 1895, p. 335.

<sup>6</sup> Cowles, H. W. Jr.: Journ. Amer. Chem. Soc., xxx, p. 1192, 1908.

<sup>7</sup> Biochem. Zeitschr., xxxv, p. 176, 1911.

<sup>8</sup> This Journal, xii, p. 268, 1912.

pyruvic acid is not precipitated nor decomposed by dilute barium hydroxide. The tissue mixtures were strained through cheese cloth and the filtrate thoroughly cooled by freezing mixtures of ice and alcohol. Barium hydroxide solution (10 per cent) previously filtered was likewise cooled and then added to the above until no further precipitate was formed. This was then rapidly filtered in the cold on a Buchner funnel. The funnel was covered to protect the solution from carbon dioxide of the atmosphere. The precipitate was thoroughly washed with ice-cold water and then together with the filter paper put into a flask for the determination of carbon dioxide as described in a previous communication.<sup>9</sup> As already mentioned, this method gave too high carbon dioxide values as pyruvic acid was carried down mechanically with the carbonate in the presence of autolytic products.

*Carbon dioxide determination under diminished pressure.* The protein solution containing pyruvic acid, with a few drops of alizarin as indicator, was placed in a double necked distilling flask arranged for vacuum distillation. The distilling flask was fitted with a capillary and also a dropping funnel for introducing phosphoric acid without opening the flask. The protruding end of the capillary was connected by rubber tubing to a series of wash bottles containing strong solutions of sodium hydroxide and finally barium hydroxide as an indicator that all air entering the capillary was freed of carbon dioxide. The further arrangement of the flasks was very similar to that used for the determination of ammonia<sup>10</sup> under diminished pressure, only, in place of the third distilling flask being attached directly to the source of vacuum, three wash bottles, two of the Drexel and one of the Wetzlar pattern were interposed. The second distilling flask was kept empty; the third flask and succeeding wash bottles contained measured amounts of  $\frac{N}{2}$  barium hydroxide. After all flasks and bottles were joined, the system was evacuated and only then was the phosphoric acid admitted by the dropping funnel to acidify the solution under examination. It was found on known carbonate solutions that the carbon dioxide was entirely removed from the flask in one hour with the corresponding formation of barium carbonate in the first two and rarely in the last two receiving flasks or bottles, and this

<sup>9</sup> This *Journal*, ix, p. 101, 1911.

<sup>10</sup> Van Slyke: *ibid.*, x, p. 20, 1911.

occurred only when large amounts of carbonate were analyzed. To avoid any error the distillation always lasted two hours. A vacuum of 15 to 20 mm. was always maintained and the flask containing the pyruvic acid never warmed beyond 25°. Care must be taken in discontinuing the vacuum that the barium hydrate containing the carbonate is not sucked back, which is best accomplished by closing the stop cock from the vacuum supply and opening the separatory funnel simultaneously. The amount of carbon dioxide admitted with the air in this way is entirely negligible. The barium hydrate is then filtered through a Gooch crucible from the carbonate and the filtrate titrated with  $\frac{N}{16}$  hydrochloric acid, using the proper precautions to exclude carbon dioxide of the air.

#### ANALYTICAL RESULTS.

Inasmuch as the results all indicate that pyruvic acid is not decomposed by aseptic animal tissues, as there was no change in the oxidation value or an increase of carbon dioxide over the controls, only a few of the numerous analyses are here recorded.

#### *Oxidation with potassium permanganate.*

*Pyruvic acid.* 1 cc.  $\frac{N}{5}$   $\text{KMnO}_4$  = 1.801 mgm. pyruvic acid. 20 cc. of a 0.2 per cent, = 0.040 mgm. pyruvic acid solution, were oxidized with 40 cc.  $\frac{N}{5}$   $\text{KMnO}_4$  and 60 cc. 10 per cent KOH. After acidifying with 50 cc. 25 per cent  $\text{H}_2\text{SO}_4$ , 18 cc.  $\frac{N}{5}$  oxalic acid were required for titration, therefore 22 cc.  $\frac{N}{5}$   $\text{KMnO}_4$  were utilized for oxidation = 0.0396 mgm. pyruvic acid.

*Pyruvic acid and leucocytes.* (1) Kept at 37° for one week. (2) Control with boiled leucocytes kept at 37° for one week. Both freed of protein with metaphosphoric acid and filtrate made up to 500 cc.

	CC. USED	$\frac{N}{5}$ $\text{KMnO}_4$	$\frac{N}{5}$ OXALIC ACID	$\frac{N}{5}$ $\text{KMnO}_4$ CC. USED	PYRUVIC ACID
		cc.			mgm.
1	20	40	17.20	22.80	41.06
2	20	40	17.30	22.70	40.88

*Pyruvic acid and rabbit kidneys.* (1) Kept at 37° for one week. (2) Control, boiled and kept at 37° for one week. Both freed of protein with metaphosphoric acid and filtrate made up to 500 cc.

	CC. USED	$\frac{N}{5}$ KMnO <sub>4</sub>	$\frac{N}{5}$ OXALIC ACID	$\frac{N}{5}$ KMnO <sub>4</sub> CC. USED	PYRUVIC ACID
		cc.			mgm.
1	20	40	17.30	22.70	40.80
2	20	40	17.10	22.90	41.40

*Pyruvic acid and rabbit kidneys.* (1) Kept at 45° for one week. (2) Control, boiled and kept at 45° for one week. Both freed of protein with metaphosphoric acid and filtrate made up to 500 cc.

	CC. USED	$\frac{N}{5}$ KMnO <sub>4</sub>	$\frac{N}{5}$ OXALIC ACID	$\frac{N}{5}$ KMnO <sub>4</sub> CC. USED	PYRUVIC ACID
		cc.			mgm.
1	20	40	14.60	25.40	45.74
2	20	40	14.50	25.50	45.92

*Determination of carbon dioxide under diminished pressure.* The carbon dioxide was always determined on the total quantity of the mixture of tissue and pyruvic acid.

- (a) Solution of sodium carbonate. 50 cc. were used.
- (b) CO<sub>2</sub> determined by steam distillation process.
- Pyruvic acid + NaOH + Henderson phosphate solution.
- (a) Pyruvic acid, 1 per cent, and rabbit kidney kept at 42° for one week.
- (b) Control, boiled and kept at 42° for one week.
- (c) Solution 3 (a) to which 25 cc. of the sodium carbonate solution mentioned above in (1) were added after the first CO<sub>2</sub> estimation was terminated.
- (a) Pyruvic acid, 0.5 per cent and rabbit kidneys kept at 37° for one week.
- (b) Control, boiled and kept at 37° for one week.
- (a) and (b) Same as 4 (a) and (b) except 1 per cent pyruvic acid.

	$\frac{N}{10}$ Ba(OH) CC. USED	$\frac{N}{10}$ HCl CC. USED	DIFFERENCE	MGm. CO <sub>2</sub>
1 (a)	78.00	61.50	16.50	36.30
(b)	60.00	43.30	16.70	36.70
2	20.00	20.00		
3 (a)	24.00	22.60	1.40	3.08
(b)	24.00	22.80	1.20	2.68
(c)	60.00	51.80	8.20	18.04
4 (a)	22.80	20.00	2.80	6.16
(b)	22.80	20.00	2.80	6.16
5 (a)	22.80	20.40	2.40	5.28
(b)	22.80	20.40	2.40	5.28



## THE FATE OF *l*-ALANINE IN THE GLYCOSURIC ORGANISM.

By H. D. DAKIN AND H. W. DUDLEY.

(From the Herter Laboratory, New York.)

(Received for publication, March 14, 1914.)

It is well established that the administration, to an animal rendered glycosuric by means of phlorhizin, of either alanine or lactic acid, is followed by a large increase in glucose excretion.<sup>1</sup> These experiments have been made with inactive alanine and with dextro and inactive lactic acid. Lusk and Ringer obtained "extra glucose" corresponding to 92 per cent of the theoretical amount possibly formed from the alanine administered while Lusk and Mandel obtained somewhat lower results with inactive lactic acid. In connection with the theory of intermediary metabolism, it became of importance to determine whether *l*-lactic acid and *l*-alanine were capable of yielding glucose in the same way as their naturally occurring enantiomorphs. We have recently published experiments showing that *l*-lactic acid is, as a matter of fact, converted into glucose in the glycosuric animal and that the same is true of methyl glyoxal which may be regarded as a possible product of the intermediary metabolism of both lactic acid and glucose.<sup>2</sup>

The object of the present communication is to record an experiment we have made upon the fate of *l*-alanine when given to a dog rendered fully glycosuric by means of phlorhizin. We find that *l*-alanine like *l*-lactic acid furnishes glucose freely under these conditions. On administering 8.5 grams of pure *l*-alanine we obtained an excretion of "extra glucose" corresponding to 7.2 grams. The amount of glucose theoretically capable of being

<sup>1</sup> For literature see Lusk "Phlorhizinglycosurie," *Ergeb. d. Physiol.*, xii, p. 315, 1912.

<sup>2</sup> This *Journal*, xv, p. 127, 1913.

formed from 8.5 grams of *l*-alanine is 8.6 grams, so that the conversion in the above experiment was almost complete.

Assuming, as seems most reasonable, at any rate until evidence to the contrary is presented, that the increased glucose excretion originates from the substance administered, we are forced to the conclusion that in the synthesis of glucose from alanine and lactic acid the asymmetry of the central carbon atom is lost. Otherwise, the synthesis of the same optically active glucose from both *d*- and *l*-alanine and both *d*- and *l*-lactic acid appears impossible of explanation. The conclusion that one of the first stages in the transformation of lactic acid and alanine into glucose involves a loss of the optical activity in the former substances appears well based. As to the nature of the symmetric product or products of intermediary metabolism, it is impossible to speak with certainty. From the fact that methyl glyoxal gives glucose in the glycosuric organism and that enzymes in animal tissues may convert it into lactic acid and furthermore the fact that by simple chemical means we have obtained methyl glyoxal from alanine, lactic acid and glucose, leads us to suspect that methyl glyoxal may be the symmetrical substance in question. Recently Embden has suggested the possibility of dihydroxyacetone being an intermediary substance in the conversion of *l*-lactic acid into glucose. A definite decision as to the mechanism of the reaction must await further experiments.

#### EXPERIMENTAL.

The conditions of the experiment were precisely similar to those adopted in previous work.<sup>3</sup> Phlorhizin (1 gram) suspended in olive oil was given daily and urine was collected in twelve-hour periods after the preliminary period of preparation was over. The *l*-alanine was given by subcutaneous injection of its aqueous solution and was readily absorbed. The dog used for the experiment weighed 9 kgm. and was in excellent condition throughout the experiment. Only one experiment was made, but the results were so decisive that it seemed hardly necessary to devote a considerable amount of additional work to the somewhat laborious task of preparing further quantities of *l*-alanine.

<sup>3</sup> This *Journal*, xiv, p. 321, 1913.

The *l*-alanine was prepared by the resolution of inactive alanine by means of a combination of the methods of Fischer,<sup>4</sup> and Pope and Jackson.<sup>5</sup> The inactive alanine was converted into benzoyl-alanine by means of benzoyl chloride and sodium bicarbonate and the purified substance (2 mols.) together with strychnine (1 mol.) and potassium hydroxide (1 mol.) were dissolved in boiling water. On cooling the solution and inoculating with a crystal of the strychnine salt of *d*-benzoylalanine<sup>6</sup> almost the whole of the dextro component crystallized out as the strychnine salt while the *l* compound remained in solution as potassium salt.

Crude *l*-benzoylalanine was obtained from the filtrate by first removing traces of strychnine with potash and then acidifying with hydrochloric acid. The *l*-benzoylalanine was completely separated from any of the dextro substance by conversion into the characteristic crystalline brucine salt, which was finally decomposed in the usual fashion. The *l*-benzoylalanine was hydrolyzed with hydrochloric acid (20 per cent) and the benzoic acid removed by steam distillation. On evaporating the clear aqueous solution to dryness, crystalline *l*-alanine hydrochloride was obtained and this was converted into the free amino-acid by means of lead hydroxide according to Fischer's directions. The substance used for injection was a well crystallized optically pure preparation. The yield of active alanines obtained by the foregoing method is satisfactory. Thirty grams of inactive alanine gave 9 grams of *d*-alanine and 10 grams of *l*-alanine.

The results of the experiment are recorded in the following table. Adopting G:N = 3.25 as the dominant value, it appears that the administration of 8.5 grams of *l*-alanine resulted in the excretion of 7.3 grams of glucose.

<sup>4</sup> Chem. Ber., xxxii, p. 2451, 1899.

<sup>5</sup> Trans. Chem. Soc., ci, p. 939, 1912.

<sup>6</sup> Unless a crystal of the salt is used for starting crystallization, the strychnine salt in spite of its being sparingly soluble in water, may remain in solution at 0° for weeks. If a crystal of the substance is not available, it may be most readily obtained by treating inactive benzoylalanine with brucine (2 mols.) to remove the easily-crystallizing *l*-benzoylalanine salt. On recovering the crude *d*-benzoylalanine from the filtrate in the usual way, no difficulty will be experienced in preparing its strychnine salt, as under these conditions it crystallizes readily.

feeding carbohydrates. The latter, as pointed out in an earlier paper,<sup>3</sup> lessens the total nitrogenous metabolism, without interfering with the efficiency of the benzoic acid to cause the production of glycocoll.

In the present investigation an attempt is made to ascertain three points:

1. The effect of feeding a simple amino-acid (alanine), alone and together with benzoic acid, upon the general protein metabolism and the formation of glycocoll.

2. The effect of feeding benzoyl-alanine upon the same phenomena.

3. The mechanism involved in the metabolism of a benzoylated nitrogen-free substance, and the formation of glycocoll.

As in our previous experiments, the tests were made on rabbits. Owing to the peculiar nature and the slight solubility of the benzoyl compounds (benzoyl-alanine and benzoyl-glucose) these substances were administered in powder form, *per os*. Certain errors may have crept in from this method of experimentation, but the uniformity of the results obtained justifies the belief that errors, if any, are negligible.

The first experiment comprises two series of nine three-day periods, in which the effect of benzoic acid and *L*-alanine singly and jointly was studied (condensed tables 1 and 2). The effect of feeding benzoyl-alanine was also tested.

As in our previous experiments, so too in the present tests it was found that the simple administration of benzoic acid causes a marked rise in the elimination of hippuric acid (tables 1 and 2, period 2). The administration of alanine alone causes a rise in the total nitrogen output (equivalent to the nitrogen of the alanine), but is without effect upon the hippuric acid output (cf. periods 3 and 4, tables 1 and 2). The simultaneous administration of alanine and benzoic acid causes a greater rise in the total urinary nitrogen, than that caused by alanine alone. The increment is equivalent to the sum total of the alanine-nitrogen and the extra nitrogen eliminated as hippuric acid (tables 1 and 2, period 6). Although the hippuric acid output in this last period (table 1, period 6) is greater than it is in the control benzoic acid period (2), i.e., 0.141 as compared with 0.122 gram of nitrogen, the difference

<sup>3</sup> Epstein and Bookman: this *Journal*, x, p. 353, 1911.

CONDENSED TABLE 1.  
Daily average for each period.

PERIOD	WEIGHT grams	FOOD (CARROTS)	BENZOIC ACID	ALANINE gram	ALANINE N	BENZOYL ALANINE grams	BENZOIC EQUIVA- LENT	URINE		
								Total N	Hippuric N	Benzoic equivalent
		grams	grams	gram	gram	grams		grams	gram	grams
1	2050	300						1.166	0.010	0.083
2	2050	300	2.0					0.934	0.122	1.059
3	2160	300						0.770	0.016	0.136
4	2100	300		1.0	0.149			0.917	0.023	0.203
5	2140	300						0.719	0.011	0.093
6	2140	300	2.0	1.0	0.149			1.094	0.141	1.233
7	2000	300						0.545	0.022	0.191
8	1950	300				2.9	1.885	1.111	0.120	1.044
9	1550	300						1.081	0.025	0.217

Animal died accidentally.

CONDENSED TABLE 2.  
Daily average for each period.

PERIOD	WEIGHT grams	FOOD (CARROTS)	BENZOIC ACID	ALANINE gram	ALANINE N	BENZOYL ALANINE grams	BENZOIC EQUIVA- LENT	URINE		
								Total N	Hippuric N	Benzoic equivalent
		grams	grams	gram	gram	grams		grams	gram	grams
1	2000	300						0.585	0.017	0.151
2	2000	300	2.0					1.190	0.132	1.146
3	2020	300						0.991	0.016	0.146
4	2000	300		1.0	0.149			1.066	0.018	0.153
5	1960	300						0.583	0.009	0.077
6	1960	210	2.0	1.0	0.149	2.9	1.885	1.344	0.115	1.002
7	1940							1.539	0.051	0.450

is not sufficient to allow any other interpretation, as is shown by the results of the corresponding experiment in table 2.

After another interval of three days benzoyl-alanine was fed to the animal. This substance was given in amounts of 2.9 grams daily,<sup>4</sup> which we found to contain an amount of nitrogen equivalent to that of the alanine of the control tests.

The rise in the total urinary nitrogen in this period (8) corresponds with that obtained in period 6, table 1, in which benzoic acid and alanine were given simultaneously. The amount of hippuric acid, however, is less than in the control period (6, table 1) but corresponds to the period in which benzoic acid alone was given. It appears from this experiment that the benzoyl alanine does not produce more glycocoll than a corresponding amount of benzoic acid. It may therefore be said that alanine, free or in combination with the benzoyl radical, does not increase the output of glycocoll. The results show also that the benzoyl radical in benzoyl-alanine (unlike that of the benzoyl-leucine) fails to produce a greater output of hippuric acid than benzoic acid.

In order to test the efficiency of the benzoyl radical in a benzoyl compound to cause the production of glycocoll, the following experiment was performed. A rabbit, weighing 2400 grams, was given 2.0 grams of benzoic acid as sodium benzoate, daily, for three days and the hippuric acid output determined. After a normal interval of three days the animal was given 1.1 grams of glucose daily for four days, and the quantity of hippuric acid eliminated was also ascertained. Following this the rabbit received 2.0 grams of benzoic acid and 1.1 grams of glucose daily, and the hippuric acid elimination determined. After another normal interval of three days, this rabbit received 3.1 grams of benzoyl-glucose daily for a period of three days. This quantity of the substance is equivalent to 2.0 grams of benzoic acid and 1.1 grams of glucose.

The results of this experiment show that the benzoyl compound does not possess any greater power to produce and to couple glycocoll than benzoic acid alone (condensed table 3). In fact it appears that the elimination of hippuric acid in the benzoyl period is slightly less than in the benzoic acid period.

<sup>4</sup> It was found upon analysis that 2.9 grams of benzoyl-alanine represent 1.015 grams of alanine.

CONDENSED TABLE 3.

*Daily average for each period.*

PERIOD	WEIGHT	FOOD (CARROTS)	BENZOIC ACID	GLUCOSE	BENZOYL GLUCOSE	BENZOIC EQUIVA- LENT	URINE		
							Total N	Hippuric N	Benzotic equivalent
	grams	grams	grams	grams	grams	grams	gram	gram	grams
1	2400	300					0.843	0.020	0.136
2	2430	300	2.0				0.911	0.144	1.256
3	2270	300						Urine lost	
4	2200	300		1.1			0.574	0.033	0.290
5	2300	200	2.0	1.1			0.765	0.081	0.702
6	2260	285					0.542	0.011	0.097
7	2175	300		1.03	2.9	1.86	0.714	0.039	0.338
8	2190	300					0.639	0.013	0.120

In our experiments with benzoyl-leucine previously reported, we have observed that the increased glycocoll output in the benzoyl period was greater than could be ascribed to the leucine radical. We have attributed this result to a possibly greater efficiency of the benzoyl radical to couple glycocoll. It appears now from the experiment described above that the results obtained in the previous experiments were probably due to the presence of a greater amount of available glycocoll.

## SUMMARY.

The data obtained in these experiments show that alanine, free or combined with a benzoyl radical, fails to yield glycocoll. This result is in accord with work of Magnus-Levy.<sup>5</sup> From the structural character of alanine it is not conceivable that it can be directly converted into glycocoll. Our results serve to show, however, that in its decomposition in the body, alanine does not yield cleavage products which can be synthesized into glycocoll. Furthermore, alanine has no direct or indirect effect on hippuric acid metabolism.

The increased output of hippuric acid, and hence glycocoll, observed after the administration of benzoyl-leucine is probably not a function of the benzoyl radical of the compound.

<sup>5</sup> Magnus-Levy: *loc. cit.*

TABLE 1.

DATE	WEIGHT	FEED CARBOTS	BENZOIC ACID	ALANINE	ALANINE N	BENZOYL ALANINE	BENZOIC EQUIVA- LENT	ALANINE N EQUIVA- LENT	DAILY TOTAL N	TOTAL N FOR PERIOD	TOTAL HIPPOURIC N FOR PERIOD	EXTRA HIPPOURIC N	BENZOIC EQUIVA- LENT OF HIPPOURIC ACID FORMED
		grams	grams	gram	gram	grams	grams	gram	grams	grams	gram	gram	grams
Nov. 10-11	2040	300							1.169				
11-12	2070	300							1.113	3.500	0.029		0.250
12-13	2070	300							1.218				
14-15	2010	300	2.0						1.114				
15-16	2040	300	2.0						0.791	2.801	0.365	0.336	3.176
16-17	2100	300	2.0						0.896				
17-18	2180	300							0.616				
18-19	2170	300							0.840	2.310	0.048	0.019	0.410
19-20	2140	300							0.854				
20-21	2100	300		1.0	0.149				0.987				
21-22	2100	300		1.0	0.149				1.036	2.751	0.070	0.041	0.610
22-23	2100	300		1.0	0.149				0.728				
23-24	2140	300							0.896				
24-25	2100	300							0.490	2.156	0.032	0.003	0.280
25-26	2180	300							0.770				
26-27	2130	300	2.0	1.0	0.149				1.029				
27-28	2120	300	2.0	1.0	0.149				1.106	3.281	0.424	0.395	3.690
28-29	2070	300	2.0	1.0	0.149				1.146				
29-30	2020	300							0.560				
30-1	2000	300							0.525	1.645	0.066	0.037	0.574
Dec. 1-2	1970	300				2.9	1.885	0.152	0.560				
2-3	2020	300				2.9	1.885	0.152	1.750				
3-4	2000	300				2.9	1.885	0.152	0.952	3.323	0.360	0.331	3.133
4-5	1740	300				2.9	1.885	0.152	0.630				
5-6	1600	300							0.630				
6-7	1540	300							1.533	2.163	0.050		0.435

TABLE 2.

OATE	WEIGHT	FEED CARBOXS	BENZOIC ACID	ALANINE	ALANINE N	BENZOYL ALANINE	BENZOIC EQUIVALENT	ALANINE N EQUIVALENT	DAILY TOTAL N	TOTAL N FOR PERIOD	TOTAL HIPURIC N FOR PERIOD	EXTRA HIPURIC N	BENZOIC EQUIVALENT OF HIPURIC ACID FORMED
	grams	grams	grams	gram	gram	grams	grams	gram	grams	grams	gram	gram	grams
Nov. 11-12	2000	300							0.609				
12-13	2020	300							0.490				
13-14	1950	300							0.553				
14-15	2040	300							0.420	2.765	0.088		
15-16	2000	300							0.693	1.659	0.052*		0.453
16-17	2020	300	2.0						1.120				
17-18	300	300	2.0						1.176	3.570	0.396	0.344	3.447
18-19	2080	300	2.0						1.274				
19-20	2070	300							0.952				
20-21	2000	300							0.938	2.975	0.050		0.440
21-22	2000	300							1.085				
22-23	2000	300	1.0	0.149					0.854				
23-24	1970	300	1.0	0.149					1.064	3.198	0.055		0.460
24-25	300	300	1.0	0.149					1.280				
25-26	1920	300							0.560				
26-27	2000	300							0.595	1.749	0.028		0.230
27-28	1920	300							0.594				
28-29	2000	300	2.0	0.149					1.394				
29-30	1920	300	2.0	0.149					lost	2.688	0.346	0.294	3.007
30-1	1980	30	2.0	0.149					1.294				
Dec. 1-2	2020	220							0.560				
2-3	2020	300							0.574	1.674	0.081	0.039	0.700
3-4	1920	300							0.560				
4-5	1940					2.9	1.885	0.152	0.539		0.051		0.450

\* For three days.

TABLE 3.

DATE	WEIGHT grams	FEED CARROTS grams	BENZOIC ACID grams	GLUCOSE grams	BENZOYL GLUCOSE grams	BENZOIC EQUIVA- LENT grams	DAILY TOTAL N grams	TOTAL N FDR PERIOD grams	TOTAL HIPURIC N gram	BENZOIC EQUIVALENT OF HIPURIC ACID FORMED grams
Nov. 14-15	2410	300					1.183			
15-16	2410	300					0.806			
16-17	2430	300					0.931			
17-18	2390	300					0.644			
18-19	2400	300					0.777	2.529	0.037	0.510
19-20	2400	300					0.630			
20-21	2400	300	2.0				0.840			
21-22	2470	300	2.0				0.910	0.433	0.433	3.767
22-23	2450	300	2.0				0.832			
23-24	2340	300								
24-25	2240	300								
25-26	2220	300		1.1			0.549	2.369		
26-27	2210	300		1.1			0.630			
27-28	2140	300		1.1			0.497	1.722*	0.100*	0.870
28-29	2200	300		1.1			0.693			
29-30		300	2.0	1.1			0.553			
30-1	2320	150	2.0	1.1			0.889	2.206	0.242	2.105
Dec. 1-2		150	2.0	1.1			0.854			
2-3	2370	260					0.463			
3-4	2380	300					0.651	1.625	0.034	0.290
4-5	2220	300					0.511			
5-6	2220	300					0.553			
6-7		300		1.1	3.1	2.0	0.791	2.142	0.117	1.017
7-8	2130	300		1.1	3.1	2.0	0.798			
8-9	2170	300		0.9	2.5	1.613	0.633	1.918	0.038	0.321
9-10	2200	300					0.469			
10-11	2200	300					0.791			

\* For three days.

## ON THE PREPARATION OF CREATINE, CREATININE AND STANDARD CREATININE SOLUTIONS.

By OTTO FOLIN.

(From the Biochemical Laboratory of Harvard Medical School, Boston.)

(Received for publication, March 17, 1914.)

In order to enlarge the scope of the colorimetric method for the determination of creatinine and creatine it is, I think, necessary to replace the bichromate standard with standard creatinine solutions, and in the next two papers will be described various adaptations of the method based on this principle. In connection with this work I have again gone over the subject of the preparation of creatine and creatinine from urine in order to render more easy their preparation on a large scale as well as to facilitate the preparation of standard creatinine solutions.

*The precipitation of creatinine from urine.* The precipitate obtained on adding picric acid to urine consists to a very large extent of the double picrate of creatinine and potassium ( $C_4H_7N_3O \cdot C_6H_2(NO_2)_3OH \cdot C_6H_2(NO_2)_2OH$ ) containing 18.55 per cent creatinine. This compound is extraordinarily insoluble so that practically all the creatinine of the urine is thrown out of solution by the picric acid even if no large excess of picric acid is added. If a large excess of picric acid is added the above compound is obtained in less pure condition because potassium and sodium are also precipitated and the total amount of creatinine precipitated cannot be materially increased by this means because it is practically quantitative without it. For the precipitation of creatinine from human urine the following process is satisfactory.

To 8 liters of urine in a large precipitating jar add with stirring 60-80 grams of picric acid (8-10 grams per liter) dissolved in 400 cc. of hot alcohol and let the mixture stand over night. Remove the supernatant liquid with a siphon and wash the precipitate on a Buchner funnel with cold water.

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In the preparation of creatinine from urine it is advantageous to work with as large quantities as the available laboratory utensils will permit, but reasonably fresh and well preserved urine is necessary for the best results. Urines which have become alkaline from ammoniacal decomposition should not be used because in such urines a large proportion of the creatinine has been destroyed and because the ammonia is also precipitated by the picric acid.

*The preparation of creatinine zinc chloride from the crude picrate.* Anhydrous potassium carbonate (2 grams for 10 grams of dry picrate) may advantageously be substituted for potassium bicarbonate when setting free creatinine from the crude picrate. It dissolves quickly and decomposes the picrate at room temperatures without any foaming.

To 500 grams of the dry picric acid precipitate add 100 grams of potassium carbonate and 750 cc. of tap water. Stir for about ten minutes and let stand with occasional stirring for one to two hours. Filter on a Buchner funnel and wash the sediment two to three times with small quantities of cold water. Transfer the creatinine-containing filtrate to a very large beaker or jar and add cautiously 100 cc. of 99 per cent acetic acid (1 cc. for each gram of carbonate taken). When added in the right way, *i.e.*, by dropping on top of the foam without stirring, the strong acetic acid acts like alcohol in breaking up the foam. To the acidified wine-red solution is then added about one-fourth volume of concentrated alcoholic zinc chloride solution. An abundant precipitate of creatinine zinc chloride should come down at once. If this does not happen the reason is almost invariably that enough zinc chloride solution has not been added.<sup>1</sup>

*The decomposition of creatinine zinc chloride with lead hydroxide.* This old method yields excellent results. Nevertheless as it is possible to lose nearly all the creatinine in the process, it may be worth while to describe the procedure which in my hands has

<sup>1</sup> In preparing strong alcoholic zinc chloride solutions it is to be noted that an undissolved residue does not necessarily indicate that the solution is anywhere nearly saturated. The salt is somewhat hydrolyzed and the sediment may be zinc hydroxide which dissolves readily on the addition of a few drops of hydrochloric acid. Zinc chloride dissolves in about 1 part of alcohol.

proved satisfactory. Lead oxide cannot be substituted for the freshly precipitated compound obtained on adding alkali to a lead salt solution. The best lead salt for the purpose is the nitrate, because from this salt the hydroxide can be precipitated by the addition of an excess of ammonia. If the acetate is used a fixed alkali must be employed for the precipitation and then it must be added cautiously because of the solubility of lead hydroxide in such alkalies. Four to five grams of lead nitrate should be taken for each gram of the creatinine zinc salt to be decomposed. The nitrate is dissolved in seven to eight parts of cold water and is precipitated by the addition of about 2 cc. of strong ammonia for each gram of nitrate taken. The lead hydroxide so precipitated settles very rapidly. The supernatant liquid is removed by a siphon and the residue is washed three times with large quantities of water. The creatinine zinc chloride is added to 30 parts of water previously heated nearly to boiling and contained in a flask so large that it is not more than two-thirds filled by the water. After adding the zinc salt the mixture is heated to boiling so as to get a large part of it in solution. The lead hydroxide suspended in a small quantity of water is then added, a small portion (about one-fifth) at a time, and the mixture is boiled a few minutes after each addition. After all the lead has been added it is advisable to boil the solution for half an hour or more in order to render the insoluble residue more granular. The mixture is then cooled and filtered. The filtrate should be absolutely clear. If it is not, complete the filtration and pass hydrogen sulphide into the filtrate for one or two minutes and filter again. The clear filtrate so obtained is completely freed from lead by means of hydrogen sulphide.

The further treatment of this creatine-creatinine solution depends on whether creatinine or creatine is desired.

#### *The preparation of creatine.*

A slow but not laborious process of obtaining the maximum amount of creatine is to make use of the decomposition of a large proportion of the creatinine at low temperatures (80–90°).

The creatine-creatinine solution is evaporated on the water bath to dryness and the residue is dissolved in fifteen to twenty parts of

The most specific precipitant for creatinine is zinc chloride, and creatinine zinc chloride is, I think, the most serviceable compound for the preparation of standard creatinine solutions.

The crude creatinine zinc chloride as obtained from the crude picrate contains 5–8 per cent of impurities. By three recrystallizations, even without the use of boneblack, it assays fully 100 per cent pure when using the  $\frac{N}{2}$  bichromate solution as a standard. These recrystallizations are carried out as follows: The salt is dissolved in 10 parts boiling 25 per cent acetic acid. To the hot solution is added one-tenth its volume of concentrated alcoholic zinc chloride and one and a half volumes of alcohol. The mixture is left standing over night, filtered, and the precipitate is washed with a little alcohol. After two more recrystallizations carried out in the same way the product is pure. The compound dissolves readily in tenth-normal hydrochloric acid, and the solutions so obtained keep indefinitely—1.6106 grams dissolved in a liter of  $\frac{N}{10}$  hydrochloric acid contains 1 mgm. creatinine per cubic centimeter.

One liter of such a standard solution is adequate for several thousand creatinine determinations made according to the method to be described in the next paper.

## ON THE DETERMINATION OF CREATININE AND CREATINE IN URINE.

BY OTTO FOLIN,

WITH THE ASSISTANCE OF J. L. MORRIS.

*(From the Biochemical Laboratory of Harvard Medical School, Boston.)*

(Received for publication, March 17, 1914.)

Ever since the introduction of the colorimetric method for the determination of creatinine, potassium bichromate, in half normal solution, has been used as the standard for measuring the color obtained in the reaction of creatinine with picric acid and sodium hydroxide. That the bichromate has been serviceable for the purpose is evidenced by the fact that no other standard has ever been proposed. Potassium bichromate is, however, by no means ideal as a general standard of measure for the color comparisons involved in creatinine determinations. For determinations in ordinary urines containing 7-15 mgm. of creatinine in a volume of 5-15 cc. it may be regarded as satisfactory. For more dilute urines (or creatinine solutions) obtainable in unlimited quantities a fair degree of accuracy can be obtained with the bichromate standard by using the proportions of picric acid and alkali recommended by Shaffer.

The use of the rigid bichromate standard imposes distinct and wholly unnecessary limitations on the applications of a remarkably flexible analytical method, and now that pure creatinine compounds can be prepared from urine with very little work it is, I think, a mistake to continue the use of the bichromate except perhaps for purely routine determinations in the course of ordinary urine analysis. The technique described in this paper is based on the use of 1 mgm. of creatinine as a standard in the making of the color comparisons.

*The determination of creatinine in urine.*

With the substitution of a creatinine solution for the half normal bichromate solution as a standard in the colorimetric determination of creatinine certain additional modifications in the procedure have become necessary or desirable. The fading of the color produced when picric acid and an excess of alkali are added in the usual manner to the creatinine solution proved at first a distinct drawback to the use of the creatinine standard, for in order to avoid errors due to this fading it was necessary that the reaction should be made practically simultaneously in the standard and in the unknown. It is however possible to so make the reaction that the color does not fade in the course of twenty-four hours. If anything the color obtained is a shade stronger at the end of that time. This fact, discovered independently and simultaneously by Mr. Morris and myself, is, I think, important for it permits the use of a single standard for the whole day, and it obviates the necessity of being in any hurry in the making of the colorimetric readings.

One milligram of creatinine plus 20 cc. of saturated picric acid solution plus 1.5 cc. of 10 per cent sodium hydrate solution (added from a burette) diluted after ten minutes' standing to 100 cc. gives a highly colored, stable solution, one therefore eminently suitable for use as a standard in connection with all ordinary creatinine determinations. With the more common human urines containing from 0.5 to 1.5 mgm. of creatinine in 1 or 2 cc. the process of making the color comparison is so simple that it is hardly possible to make a mistake—provided only that the 10 per cent alkali is measured out with a burette so that the same amount within 0.1 or 0.2 cc. is added to both the standard and the unknown.

One cubic centimeter of the standard creatinine solution is measured into a 100-cc. volumetric flask and 1 cc. of the urine into another; 20 cc. of saturated picric acid solution (measured with a cylinder) are added to each and then the alkali, 1.5 cc. of 10 per cent solution. At the end of ten minutes the flasks are filled up to the mark with tap water and the color of the unknown is determined. It makes little difference whether the standard is set at 10, 15 or 20 mm., 10, 15 or 20 divided by the reading of the unknown gives in milligrams the amount of creatinine present in the volume of

urine taken. If the urine reads less than two-thirds or more than one and one-half that of the standard the determination should be repeated with more or with less urine.

The only special apparatus needed besides the colorimeter is accurate 1-cc. pipettes of the kind described in this *Journal* a short time ago.<sup>1</sup> One precaution should perhaps be mentioned. Those not used to the small pipettes referred to will be apt to conclude that by diluting the standard creatinine solution so that 5 cc. contains 1 mgm. they can just as well use 5-cc. pipettes and then work with 5 cc. of urine and dilute the resultant colored solution to 500 cc. while the standard is diluted to 100. To do so would be to introduce a very considerable error. Those who wish to use 5- or 10-cc. pipettes had better work with diluted urines (1:4), or else they should take 5 or 10 mgm. of creatinine as the standard. In the latter case the amount of alkali taken must be increased to 5 cc. as in the original method, and a fresh standard must be used for each set of determinations because of the fading. It would doubtless be possible to find the proportions of picric acid and alkali which will give stable colored solutions with 5 and with 10 mgm. of creatinine but I have made no attempt to do so.

With dilute urines requiring more than 5 cc. to give 1 mgm. of creatinine the standard should also be diluted with a corresponding volume of water before adding the alkali.

In working with very small animals such as rats 0.5 or 0.2 mgm. of creatinine may be used as a standard as was done by J. L. Morris in our work<sup>2</sup> on whiterats. The one great advantage of using creatinine as the standard lies in the fact that the same technique is directly applicable to creatinine solutions of almost every degree of concentration provided only that the standard is varied accordingly.

In the making of colorimetric creatinine readings there is one other point which should be mentioned. It happens very often when the colorimeter prisms are immersed in the liquids to be compared that a bubble of air is caught under the prism. Unless this is prevented the readings obtained are, of course, erroneous. Moreover, in the making of an extended series of colorimetric comparisons it sometimes happens when the water is cold that a great many

<sup>1</sup>This *Journal*, xi, p. 494, 1912.

<sup>2</sup>*Ibid.*, xiv, p. 510, 1913.

very minute air bubbles escape from the liquid and gradually accumulate under the stationary prism immersed in the standard solution. A considerable amount of light is thus cut off and the comparisons become correspondingly erroneous. The simplest effective way to avoid this error is to empty the cylinder as soon as any air bubbles are noticeable on the sides and refill it from the standard solution in the flask.

*The determination of creatine in urine.*

Much work has been done to find the most suitable condition for transforming creatine quantitatively into creatinine, but the only important modification of my original procedure is the use of higher temperatures by means of the autoclave, thus shortening the time of heating from three hours to about half an hour. This modification introduced by V. C. Myers in 1907 yields rather more certain results than are obtained by heating on the water bath for three hours. The choice of acid to be used is not particularly important. Hydrochloric acid is probably the least suitable because it produces the maximum amount of coloring matters, although the error due to coloring matters may be regarded as negligible in view of the fact that the heating with acids does not produce an increase of the creatinine readings in the case of most urines obtained from normal men. The phosphoric acid recently introduced by Rose is liable to give erroneous results. The anomalous results obtained by Rose with the urine of dogs are, however, probably due only to his not having added the right amount of alkali in making the color reaction. Phosphoric acid titrates (with phenolphthalein as indicator) as a dibasic acid, but in the presence of an excess of alkali it acts as a tribasic one, and unless due allowance is made for this fact the results obtained must necessarily be too low. The uncertain and unsatisfactory results obtained by Thompson<sup>3</sup> and his associates with phosphoric acid may be similarly explained. It is not the acid but the temperature which is the important factor in the transformation of creatine into creatinine.

The substitution of the autoclave for the water bath in connection with the colorimetric determination of creatine is certainly

<sup>3</sup> *Biochem. Journ.*, vii, p. 458, 1913.

serviceable when it is a question of making a large number of such determinations at one time. But on the whole it does not seem altogether satisfactory to be dependent on it for so simple a determination. The time necessary for a creatine determination by the water-bath procedure can be materially shortened by boiling the solutions over a naked flame.

In using this procedure one can take advantage of the fact that by greatly diluting the creatine solution with water the process is hastened. Moreover, when working with about 1 mgm. of creatine and creatinine it is not necessary to add any mineral acid whatever. Instead of such acids I simply add in advance the picric which is to be used for the development of the color. The determination of creatine + creatinine in urine by this modification is as follows:

Enough urine to give 0.7–1.5 mgm. of creatinine is measured into a weighed Erlenmeyer Jena flask (cap. 200 cc.). Saturated picric acid solution (20 cc.), about 130 cc. of water and a few very small pebbles to promote even boiling are added and the mixture is gently boiled, preferably over a microburner for about one hour. At the end of this time the heat is increased and the solution is boiled down to rather less than 20 cc. The flask is transferred to the scales and enough water is added to make the total solution equal to 20–25 grams. The solution is cooled in running water, 1.5 cc. of 10 per cent sodium hydroxide are added, and the total creatinine is determined as in the preformed creatinine determination using 1 mgm. of creatinine as a standard. In my hands this procedure gives absolutely quantitative results every time with 1.32 mgm. of crystallized creatine, even in the presence of as much as 25 mgm. of urea nitrogen, and 50 mgm. of glucose, cane sugar, lactose, or levulose.

The autoclave method can also be used with picric acid as the only added acid provided that only enough urine is taken to give 0.7–1.5 mgm. of creatinine. It will not work with the old method of using 10 cc. of urine because of the large amount of ammonia set free during the heating.

In the presence of levulose, or sugars which on hydrolysis yield levulose, the autoclave process cannot be used.<sup>4</sup>

<sup>4</sup>Levulinic acid like acetoacetic ester gives a strong color with the alkaline picrate.



## ON THE DETERMINATION OF CREATININE AND CREATINE IN BLOOD, MILK AND TISSUES.

By OTTO FOLIN.

(From the Biochemical Laboratory of Harvard Medical School, Boston.)

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### *The determination of preformed creatinine in blood and milk.*

The advantage of using known creatinine solutions instead of  $\frac{N}{2}$  potassium bichromate as standards is nowhere else so decisive as in the determination of creatinine and creatine in such fluids as blood and milk where the preformed creatinine amounts to only 1-2 mgm. per 100 cc. By the help of such standard creatinine solutions the determinations become almost as simple as the corresponding determinations in urine. The method is as follows.

Ten cubic centimeters of blood or milk are measured into a 50-cc. volumetric flask, or better into a 50-cc. shaking cylinder which can be closed with a glass stopper. The flask or cylinder is then filled up to the 50-cc. mark with saturated picric acid solution and shaken a few times. About 1 gram of dry picric acid is then added to the mixture and the shaking is continued for five minutes. The mixture is then transferred to centrifuge tubes, the sediment and precipitate are shaken down and the supernatant fluid is poured through a filter. This is the most economical process where but little blood is available. When this is not the case the quantities taken may be doubled and the filtration can then be made without preliminary centrifuging process. By this treatment practically all the protein materials are removed and the creatine and creatinine are obtained in the picric acid filtrate. The filtrate is at the same time practically a saturated picric acid solution.

For the colorimetric determination of the preformed creatinine in the filtrate all that is necessary is to prepare a correspondingly dilute solution of creatinine in saturated picric acid solution. A

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solution containing 0.2 mgm. of creatinine per 100 cc. is suitable for this purpose. This can be prepared in a few moments by transferring 1 mgm. of creatinine from the standard creatinine solution used for urine work to a 500-cc. volumetric flask and then making up to volume with saturated picric acid solution. This solution can be kept on hand as the creatinine is not precipitated on standing on account of the great dilution. On adding the same amount of alkali to equal volumes of filtrate from the blood or milk and of the known picric acid solution the color produced corresponds to the amount of creatinine present, provided that neither contains more than one and one-half times as much as the other. It is absolutely essential, however, that exactly the same amount of the 10 per cent alkali should be added to each solution, because in saturated solutions of picric acid the alkali deepens the color even when no creatinine is present. The amount of alkali which I have found to yield the most reliable results when making creatinine determinations in this manner is 5 cc. of 10 per cent sodium hydrate per 100 cc. of picric acid solution.

When measuring out the alkali with an ordinary burette the simplest way to get the same amount of alkali for the unknown and the standard is to determine how many drops, as obtained from the burette, correspond to 5 cc., and then to add one-fifth of that number to 20 cc. of the unknown filtrate and to 20 cc. of the known solution. Ten cc. of the filtrate from the blood or milk may be used for the color comparison, in which case the alkali added must, of course, be only as many drops as correspond to 0.5 cc.

Sometimes the filtrate obtained from blood becomes slightly turbid after the addition of the alkali. It must then be centrifuged or filtered before using it for the color comparison.

Ten minutes' standing after the addition of the alkali is adequate for the development of the color, and the solutions are then ready for the color comparison without any further dilution. They are accordingly transferred to the cylinders of the Duboscq colorimeter and compared in the usual manner. The standard creatinine solution in this case can advantageously be set at 20 mm. because the color of the solutions are not very deep, but it is not at all essential that this should be done.

The calculation of the creatinine in the blood or milk is the same whether the standard is set at 10, 15 or 20 mm. and whether 10 or

20 cc. of the filtrate were taken for the making of the color reaction. When 10 cc. of blood are diluted to 50 cc., or 20 cc. to 100 cc., and the standard contains 0.2 mgm. of creatinine per 100 cc., according to the directions described above, the reading of the standard divided by the reading of the unknown gives without any further calculations the creatinine in milligrams contained in 100 cc. of blood (or milk).

There is only one special precaution to be noted in connection with this determination, and this has to do with the collection of the blood. In collecting blood to be used for creatinine determinations some care should be taken not to add too much potassium oxalate for the prevention of clotting. Ten drops of a 20 per cent solution is enough for 30 cc. of blood, and it is better to measure it out in this way than to use unweighed amounts of the dry salt. The reason is that the potassium of the oxalate is precipitated by the picric acid and oxalic acid is set free. This reaction is probably not quantitative, but if too much oxalate has been used the amount of acid set free is large enough to introduce a considerable variation in the amount of color obtained. If a large excess of oxalate has been added a part of the filtrate obtained from the blood must be titrated to determine its acidity compared with that of the saturated picric acid solution, and a corresponding increase must be made in the alkali added for the development of the color.

It will doubtless occur to some that the difficulty arising from use of too much oxalate might be obviated by substituting sodium or some other oxalate for the potassium salt, but I have devoted considerable time to the study of this problem and have not been able to find any more satisfactory salt than the potassium oxalate.

*The determination of creatine plus creatinine in blood and milk.*

For the determination of the so-called total creatinine in blood, milk and exudates, the preliminary precipitation with picric acid is conducted in exactly the same manner as has been described above in connection with the determination of the preformed creatinine. The filtrate obtained from 10 cc. of blood diluted with picric acid solution to 50 cc. amounts usually to rather more than 30 cc., and 10 cc. of this filtrate are all that are needed for the

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total creatinine determination. In actual practice, embracing some two hundred determinations in many kinds of blood, we first of all measure out 10 cc. of the creatine-creatinine filtrate into a small Erlenmeyer flask (capacity 25 or 50 cc.) or large test tube and then we use 10, 15 or 20 cc. of filtrate, depending on how much remains, for the determination of the preformed creatinine.

For the conversion of the creatine into creatinine we have so far used only the autoclave method. The flask or test tube containing 10 cc. of the filtrate is covered with tinfoil, transferred to the autoclave and heated to about  $120^{\circ}\text{C}$ . for about twenty minutes. When using the autoclave it is important not to open it at the end of the heating until the temperature has fallen to below  $100^{\circ}$  so as to avoid all mechanical losses.

When cooled to room temperature the solution is then rinsed into a 25-cc. volumetric flask or to the 25 cc. mark in a measuring cylinder with saturated picric acid solution and 1.25 cc. of 10 per cent sodium hydrate solution are added for the development of the color reaction.

Two standard creatinine solutions in saturated picric acid are necessary in this determination because of the variations in the creatine contents of normal blood. When working on hospital patients the variations are greater still, and three standard creatinine determinations are desirable. These standard solutions contain 0.5, 1 and 2 mgm. of creatinine respectively per 100 cc. of saturated picric acid solution.<sup>1</sup> To 20 cc. of each of these solutions in measuring cylinders is added 1 cc. of 10 per cent sodium hydrate. By inspection one can readily tell which standard comes nearest to having the same color as the unknown, and with this as a standard the color comparison is then made in the usual manner by the help of the Duboscq colorimeter. The colors of these solutions are much deeper than those obtained in the determination of the preformed creatinine, and the standard solution is consequently usually set at 10 mm.

The reading of the standard in mm. multiplied by 125 and by 0.5, 1 or 2, according to which standard is used, when divided by the reading of the unknown in mm. gives the amount of creatine + creatinine in milligrams per 100 cc. of blood or milk.

<sup>1</sup> These solutions are made by putting 1, 2 and 4 cc. of the standard creatinine zinc chloride solution in 200-cc. volumetric flasks and making up to volume with saturated picric acid solution.

*The determination of preformed creatinine in muscle and other tissues.*

For the determination of preformed creatinine in muscles and other tissues 10 grams are desirable. The tissues must be fresh, and results obtained on human autopsy materials are therefore usually worthless. The weighed tissue is transferred to a mortar (inside diameter 10–15 cm.) and is then cut into small pieces with a pair of (preferably curved) scissors. About 20 grams of sand are added and the mixture is rubbed into a fairly uniform paste. 43 cc. of saturated picric acid solution are gradually added to the paste while the rubbing is continued and finally about 1 gram of solid picric acid. The rubbing and stirring is continued for five to ten minutes after the last addition of picric acid.

By this means the proteins of muscle are converted into insoluble picrates and the creatinine stays in solution. The volume of the picric acid solution added, 43 cc., is intended to give as nearly as practicable 50 cc. of solution making allowance for the fact that about 75 per cent of the muscle is water.

The mixture is poured on a filter, 20 cc. of the filtrate are transferred to a dry measuring cylinder, and 1 cc. of 10 per cent sodium hydrate solution is added to develop the color that is to serve as a measure of the creatinine present. The picric acid creatinine solution containing 0.5 milligram of creatinine per 100 cc. is used as a standard, and to 20 cc. of it is added 1 cc. of 10 per cent sodium hydrate at the same time that the alkali is added to the muscle extract. The standard is set usually at 20 mm. and this figure multiplied by 2.5 and divided by the reading of the muscle extract gives the creatine in milligrams per 100 grams of muscle.

In all such determinations the colored solution must be filtered or centrifuged before making the color comparison.

In the case of some tissues, notably the liver and the brain, it is sometimes impossible to obtain clear filtrates suitable for creatinine determinations. By the addition of a little formalin, 2 cc. of 40 per cent solution to 10 grams of tissue, and ten minutes' standing before the extraction with picric acid solution, clear extracts are obtained. The formalin does not interfere with the subsequent determinations in such extracts. Formalin cannot be used in connection with the determination of creatine and creatinine in urine.

*The determination of creatine in muscles and other tissues.*

In all hitherto published procedures for the determination of creatine in muscles much time and labor is expended on the separation of the creatine from the protein materials. So far as I am aware there is nothing to show that such separation is necessary, and aside from the laboriousness of such a process it also undoubtedly diminishes the accuracy of the determination. For these reasons I prefer to dissolve the proteins at the same time that the creatine is being converted into creatinine, as does Baumann in the method recently published in this *Journal*.<sup>2</sup>

Baumann separates the creatinine from the protein materials before making the determination. It is, of course, possible that such a separation may be necessary, but from the character of the results which we have obtained in the course of a very large number of determinations it appears to me improbable that such is the case. Since we have found it convenient to use higher temperatures than is customary when converting creatine into creatinine by help of the autoclave, I have regarded the glycogen and the alleged decomposition of creatinine by acids as a greater source of danger than the protein materials. Creatinine is not destroyed, however, even when heated to 140°C., and glucose does not interfere at the temperature we use, 130–135°.

By the method described below the creatine in muscle can be determined in less than two hours. The method is as follows: Five grams of muscle or other tissue, cut up fine with scissors or a meatgrinder, are transferred to a 200-cc. Erlenmeyer flask and 100 cc. of  $\frac{N}{2}$  sulphuric acid are added. The flask is covered with tinfoil and is then heated in the autoclave at 130–135° for thirty to forty minutes. The tissue is almost wholly dissolved by this treatment. After cooling to below 100°C. the autoclave is opened, the contents of the flask are cooled and transferred to a 200-cc. volumetric flask. After shaking a little to break up the flocculent skeletons of the tissues the contents are diluted to 200 cc. and well mixed.

<sup>2</sup> This *Journal*, xvii, p. 15. Baumann's method was first presented at the annual meeting of the American Biochemical Society, December, 29, 1913. As I stated at that meeting I had worked out a somewhat similar process which had then been in use in my laboratory for about two months.

The solution is poured on a filter and 10 cc. of this filtrate are titrated with 10 per cent sodium hydrate with phenolphthalein as indicator. Another 10 cc. is then measured into a 100-cc. volumetric flask, and to it are added 20 cc. of saturated picric acid and enough 10 per cent sodium hydrate solution to give 1.5 cc. over and above that required for the neutralization of the sulphuric acid.

As standards for the determination of creatine in muscles we find it convenient to use solutions containing creatinine equivalent to 1 mgm. of creatine per cc. (1.389 grams of creatinine zinc chloride per liter) for striated muscle, and standards only half as strong for the determination of creatine in tissues other than striated muscles. In the former case the standard is set at 10 mm., in the latter case at 20 mm. when making the color comparisons. In either case 4000 divided by the reading of the unknown in mm. gives the creatine in milligrams per 100 grams of muscle.

In working with definite tissues of small animals it is in some cases not possible to obtain 5 grams of material nor is this necessary; 2 grams or even less can be used provided that the material is weighed out quickly so as to prevent loss of weight by the evaporation of water.



## ON THE CREATINE CONTENT OF MUSCLE.

By OTTO FOLIN AND T. E. BUCKMAN.

(From the Biochemical Laboratory of Harvard Medical School, Boston.)

(Received for publication, March 17, 1914.)

According to the trend of recent investigation particularly those of Myers and Fine,<sup>1</sup> Riesser<sup>2</sup> and others the determination of the creatine obtainable from muscle has assumed considerable importance. According to the first named investigators the creatine content of a given species is quite specific and varies within very narrow limits. The extraction methods for the determination of creatine are not very well suited for the determination of this point. In fact all determinations made on the basis of that principle represent minimum values though it may be conceded that by very careful and prolonged extraction such as those of Myers and Fine practically all the creatine is obtained.<sup>3</sup>

Our determinations were made according to the method described by Folin in the preceding paper.<sup>4</sup> The nitrogen was determined in 25 cc. or a corresponding proportion of the mixture obtained after hydrolyzing the muscle in the autoclave. The nitrogen and the creatine represent therefore always the same sample of muscle.

<sup>1</sup> This *Journal*, xiv, p. 9, 1913.

<sup>2</sup> *Zeitschr. f. physiol. Chem.*, lxxxvi, p. 415, 1913.

<sup>3</sup> See, however, Myers and Fine: this *Journal*, xiv, p. 15, 1913. After having made six extractions they added a "correction of 5 mgm." for the amount obtained in the seventh. See also Beker: *Zeitschr. f. physiol. Chem.*, lxxxvii, p. 23, 1913.

<sup>4</sup> The color comparisons were made independently by each of us and when any material discrepancy in our readings was obtained we made the final determinations together. In connection with these comparisons we have found that the old style Duboscq colorimeters (with moveable prisms) give somewhat erroneous results because of the varying distance between the bottom of the prisms and the mirror below. This is evidently the reason why in the newer instruments the platforms supporting the cups have been made moveable while the prisms remain fixed.

TABLE I.  
*Creatine content of muscle.*

CAT		RABBIT		DOG		HEN		TURTLE	
Creatine mg. per 100 gms.	Per cent of N as Creatine	Creatine mg. per 100 gms.	Per cent of N as Creatine	Creatine mg. per 100 gms.	Per cent of N as Creatine	Creatine mg. per 100 gms.	Per cent of N as Creatine	Creatine mg. per 100 gms.	Per cent of N as Creatine
444	4.7	571	5.7	488	4.4	571	4.9	236	4.3
580	5.7	417	3.9	435	3.9	534	4.2	292	4.3
480	5.7	548	5.3	431	3.8	548	4.9	321	4.3
460	4.7	488	4.6	408	3.5	500	4.3	333	4.3
455	4.2	556	5.0	444	3.8			339	4.7
465	4.6	488	4.4	440	3.6				
520	4.8	500	4.7						
520	4.8								
556	5.6								
421	3.5								
550	4.0								
500	4.9								
421-580	3.5-5.7	417-571	3.9-5.7	408-488	3.5-4.4	500-571	4.2-4.9	236-339	4.3-4.7

TABLE II.  
*Creatine content of heart muscle.*

CAT		RABBIT		DOG		SHEEP		HEN		TURTLE	
Creatine mg. per 100 gms.	Per cent of N as Creatine	Creatine mg. per 100 gms.	Per cent of N as Creatine	Creatine mg. per 100 gms.	Per cent of N as Creatine	Creatine mg. per 100 gms.	Per cent of N as Creatine	Creatine mg. per 100 gms.	Per cent of N as Creatine	Creatine mg. per 100 gms.	Per cent of N as Substance
221	2.9	256	3.2	327		333	4.5	190		104	2.2
333	2.8	201	2.6	307	4.3	208	4.6	166	2.5	98	1.7
240	2.7	186	2.4	323	3.8	339	5.4			100	
274	3.0	206	2.3	308	3.5	258	3.5			109	1.7
333	3.7	291	3.2	210	2.6	274	3.5			70	
294	3.4	195	2.6	250		292	4.1				
333	3.3	226	2.3			282	3.8				
250	3.0					280	3.8				
222	2.5										
233	2.8										
250	3.1										
222	2.7										
222-333	2.7-3.7	195-291	2.3-3.2	210-327	2.6-4.3	274-339	3.5-5.4			98-109	1.7-2.2

The results of our determinations indicate that the creatine contents of the muscles of cats, rabbits, and hens vary within substantially the same limits. The variations found appear to be too large to permit the use of average figures in calculations as to the alleged relationship between the creatinine elimination and the total amount of creatine in the tissues.

## ON THE CREATININE AND CREATINE CONTENT OF BLOOD.

BY OTTO FOLIN AND W. DENIS.

*(From the Biochemical Laboratories of the Massachusetts General  
Hospital and Harvard Medical School, Boston.)*

(Received for publication, March 17, 1914.)

The number of creatinine and creatine determinations in blood recorded in the literature is exceedingly small, the reason being that heretofore no suitable technique has been available. It therefore appeared to us worth while to make a series of such determinations in the blood of different animals and particularly in human blood in order to learn whether in pathological conditions there is any specific retention of creatinine similar to the specific retention of uric acid in gout. In this connection we have been especially interested in seeing how much creatinine accumulates in uremic conditions associated with extremely high retention of total non-protein nitrogen in the blood. Whenever the amount of blood available was sufficient we have determined all the common nitrogenous (non-protein) constituents in order to furnish a more complete picture of the composition of human blood.

We may state at once that although we have up to date examined the blood of about two hundred different hospital patients we have not yet been able to find any specific creatinine retention. The figures obtained indicate that the human kidneys remove the creatinine from the blood with remarkable ease and certainty. The completeness of the creatinine excretion is in fact exceeded only by the still more complete removal of the ammonium salts. Except in extreme conditions of retention approaching anuria the creatinine in the blood remains at the normal level.

In view of this outcome it does not seem necessary to make a full record of our analyses or to describe any cases in detail. Tables 1-3 representing observations on the blood of about sixty-five different patients give we believe an adequate account of the

retention of creatinine in human blood. Table 4 contains summaries of the creatinine and creatine contents found in the blood of some of the more common domestic animals. Except in the case of birds these figures are not materially different from the figures obtained for human blood.<sup>1</sup>

The absence of creatinine from the blood of birds confirms Paton's discovery that creatinine is not a normal waste product in these animals. The somewhat high creatine figures obtained from the blood of birds tend perhaps to also confirm Paton's observation that creatine takes the place of creatinine as a waste product though this point stands in need of further investigation.

TABLE 1.

*The accumulation of nitrogenous waste products (including total "creatine") in human blood.*

NO.		NON-PROTEIN N	UREA N	AMMONIA N	URIC ACID	CREATININE	CREATININE AND CREA- TINE
Dr. P.	Normal purine-free high nitrogen diet urinary nitrogen 24 gms.....	34	16	0.1	2.5	1.1	9.5
Dr. M.	Normal (?) urinary nitrogen 17 gms.....	37	18	0.1	3.0	1.2	8.5
Dr. P.	Normal, low nitrogen diet. Urinary nitrogen 6.2 gms.	32	15	0.14	2.0	1.3	6.5
Dr. M.	Normal. Urinary nitrogen 4.5 gms.....	24	11	0.11	2.0	1.4	6.9
107	Alcoholic gastritis, spinal arthritis.....	34	16	0.06	2.0	1.3	9.5
138	Mitral stenosis.....	30	14	0.09	4.5	1.2	7.0
142	Toxemia 3 weeks after delivery.....	26	12	0.1	4.4	0.9	6.0
144	Cardio-renal case, marked decompensation.....	26	12	0.5	2.8	1.8	10.0
106	Arthritis (chronic).....	30	15	0.7	3.3	1.0	8.0
123	Typhoid fever temp. 104° (second week).....	38	19	0.08	2.0	1.4	10.0
122	Typhoid temperature 104° (second week).....	33	16	0.18		1.4	8.5
147	Cystinuria.....	28	14		1.4	1.2	10.0

<sup>1</sup> The analytical methods used in these determinations are described in this *Journal*, xi, p. 527, 1912; xiii, p. 469, 1913; xvii, p. 475, 1914.

TABLE 2.

*The accumulation of nitrogenous waste products (including total "creatinine") in human blood.*

(Milligrams per 100 grams of blood).

NO. AND SEX	DIAGNOSIS	NON-PROTEIN N	UREA N	AMMONIA N	URIC ACID	CREATININE	CREATININE AND CREA- TINE
78 m.	Cardio-renal case, marked decompensation.....	326	266		4.4	1.4	8.3
82 m.	Uremia.....	288	222		9.5	31.0	
97 m.	Uremia.....	284	228	0.66	6.6	26.0	46.0
76 f.	Uremia, anuria, died two days later.....	228	180		7.5	32.0	36.0
48 f.	Uremia.....	212	132			4.0	20.0
111 f.	Uremia, convulsions.....	200	160	1.0	8.0	10.0	27.0
77 m.	Chronic nephritis.....	200	140			7.5	13.0
103 f.	Uremia, anuria.....	148	110	1.0	6.5	10.6	28.0
83 f.	Uremia.....	136	110			20.8	25.0
28 f.	Uremia, convulsions.....	125	89		10	6.2	
29 f.	Nephritis following eclampsia.....	100	76		4.1	1.0	8.2
7 m.	Uremia convulsions.....	88	70	1.0	3.5		6.6
93 m.	Acute infection.....	80	56	0.1	6	1.0	6.5
102 m.	Nephritis, hemiplegia, coma.....	80	56	0.8	4.0	3.0	14.2
14 m.	Enlarged spleen anemia...	76	41	0.25		1.0	14.6
2 m.	Age 10. Uremia, convul- sions, oedema.....	74	52	0.66	4.0		6.0
58 m.	Pneumonia (before crisis)..	72	44		5.0	1.5	20.0

TABLE 3.

*Creatinine and "creatine" in miscellaneous human blood.*  
(Milligrams per 100 grams of blood).

DIAGNOSIS AND REMARKS	NON-PROTEIN N	CREATININE	CREATINE AND CREA- TININE	DIAGNOSIS AND REMARKS	NON-PROTEIN N	CREATININE	CREATINE AND CREA- TININE
Under nutrition.....	62	0.8	5.6	Syphilitic aortitis...	44	2.3	15.0
Nephritis.....	60	1.2	8.0	Neurasthenia.....	44	1.0	8.0
Convalescent from acute nephritis.....	56	1.0	8.0	Nephritis.....	43	1.3	7.6
Nephritis.....	55	1.2	8.5	Iritis (gout?).....	40	1.2	8.2
Unknown.....	55	1.0	10	Diabetes.....	40	1.5	8.3
Rheumatism, pleurisy..	54	1.0	6.6	Nephritis.....	38	1.3	9.0
"Uremia".....	53	2.6	6.7	Tape worm.....	36	0.9	10.0
Secondary anemia, haemorrhoids.....	52	1.2	6.7	Cerebral embolism..	34	2.5	13.0
Age 75, pneumonia: died 2 days later.....	52	2.2	15.0	Cirrhosis of liver....	34	1.3	8.1
Nephritis.....	50	1.0	9.6	Double renal stone..	32	1.3	9.0
Nephritis; cirrhosis of liver.....	50	1.0	8.2	Lead poisoning.....	32	1.3	8.0
Cardiac weakness (gout?).....	50	1.2	9.0	Tonsillitis.....	30	2.5	7.0
Splenic anemia.....	50	1.3	9.0	Hodgkin's disease..	25	1.0	7.5
Nephritis.....	50	4.0	9.7	Pernicious anemia...	28	1.0	7.0
Gastric ulcer.....	48	2.2	13.0	Lipomatosis.....	30	0.8	10.0
Graefes disease.....	46	1.0		Early HgCl poison- ing.....	24	1.4	8.0
Pneumonia after crisis.	46	1.6	10.0	Renal tuberculosis...	28	1.4	8.5
Pyelonephritis.....	45	1.2	6.2				
Mild diabetes.....	45	1.2	6.0				
Arteriosclerosis.....	45	1.3	0.6				
Acute endocarditis.							
102°.....	44	2.0	12.0				

TABLE 4.

*Creatinine and total "creatinine" in the blood of some domestic animals.*  
(The figures are given in milligrams per 100 grams of blood.)

	CREATININE	CREATININE + CREATINE
Beef blood .....	2	11
Sheep blood.....	1.2	9
Pig blood.....	1.3	9
Cat blood.....	1.2	8
Rabbit blood.....	1.0	10
Hen blood.....	0.1±	11
Pigeon blood.....	0.1±	11
Goose blood.....	0.1±	12



# PROTEIN METABOLISM FROM THE STANDPOINT OF BLOOD AND TISSUE ANALYSIS.

SEVENTH PAPER.

## AN INTERPRETATION OF CREATINE AND CREATININE IN RELATION TO ANIMAL METABOLISM.

By OTTO FOLIN AND W. DENIS.

(From the Biochemical Laboratories of Harvard Medical School and the  
Massachusetts General Hospital, Boston.)

(Received for publication, March 17, 1914.)

With the observation made independently by Folin<sup>1</sup> and Klercker<sup>2</sup> (1906) that the administration of creatine is not accompanied by an appreciable increase in the creatinine elimination came the problem of explaining why these two substances so nearly related chemically seem to be so distinct and independent of each other in the metabolism, and what is the significance of the large accumulation of creatine in the muscles. To be sure the validity of the observation has been questioned. Paton,<sup>3</sup> Pekelharing, van Hoogenhuyze and Verploegh,<sup>4</sup> Myers and Fine,<sup>5</sup> and others support with more or less emphasis the idea that administered creatine is in part converted into creatinine, though all, except perhaps Paton, are agreed that the increase in the creatinine output obtained in response to creatine feeding is very small indeed.

It does not seem worth while to make an issue of the exact extent to which such an increase in the creatinine elimination under the influence of creatine feeding does occur or whether the increases observed by some of the above-mentioned investigators

<sup>1</sup> Hammarsten's *Festschrift*, 1906.

<sup>2</sup> *Beitr. z. chem. Physiol. u. Pathol.*, viii, p. 59, 1906.

<sup>3</sup> *Journ. of Physiol.*, xxxix, p. 486, 1910.

<sup>4</sup> Van Hoogenhuyze and Verploegh: *Zeitschr. f. physiol. Chem.*, lvii, p. 161, 1908; Pekelharing, and van Hoogenhuyze: *Zeitschr. f. physiol. Chem.*, lxix, p. 395, 1910.

<sup>5</sup> This *Journal*, xvi, p. 176, 1913.

are greater than could fairly be accounted for on the basis of unimportant and accidental circumstances. It is not probable that an adequate explanation of the different behavior of food creatine and tissue creatine can be obtained on the basis of the small and uncertain variations in the elimination of creatinine. The explanation recently advanced by Myers and Fine<sup>6</sup> is interesting and ingenious. These authors have attempted to show that the proportion of food creatine converted into creatinine while passing through the animal organism is as large as is the proportion of the body creatine which is daily converted into creatinine. They also have endeavored to prove experimentally that the creatine in an animal is a fixed quantity, so that by fasting or partial fasting there is a reduction of the amount in the body corresponding to the loss incurred by the elimination of creatinine (and creatine) in the urine, and that therefore the whole of the creatine present in the animal at the beginning of such an experiment (and no more) can be accounted for at every stage of the fast. Novel and interesting as the work of Myers and Fine undoubtedly is it proves rather too much. It does not seem entirely plausible that fasting or partially fasting animals should not form new precursors of creatine and creatinine out of other nitrogenous materials, since that is exactly what fed animals, including herbivorous animals, must be doing all the time. Should further investigations substantiate the findings and conclusions of Myers and Fine the results must be regarded as very important.

In 1907 Hofmeister suggested in a paper published by Urano<sup>7</sup> that the creatine might be a part of the muscle protoplasm, and Urano made some experiments the results of which indicated that creatine in the living muscles of frogs is present in a non-dialyzable form. While no one has disputed Urano's findings, so far as we know the work has never been repeated nor have either the results or the hypothesis which gave rise to the work attracted any considerable attention. The technique employed by Urano was rather crude. He did not use a colorimeter of any kind for the creatinine estimations.

The idea that creatine might be held in some sort of combination with the muscular protoplasm has probably been in the minds

<sup>6</sup> This *Journal*, xv, p. 283, 1913.

<sup>7</sup> *Beitr. z. chem. Physiol. u. Pathol.*, ix, p. 104, 1907.

of most workers on the subject. But it is clear that no one has heretofore made serious use of it as a working hypothesis. Indeed it has seemed too hypothetical, too far removed from tangible experimental results.<sup>8</sup> The remarkable accumulation of creatine in vertebrate muscles is not more remarkable than the still greater accumulation of urea in the blood and muscles of sharks, and one would hesitate to assume that such urea is an integral part of the protoplasm, however ready one may be to concede that it performs some useful function.

Creatine must, however, be a waste product or a synthetic product serving some special function, or as a synthetic product it may in fact be a part of the active living protoplasm. We believe that the last named alternative represents the facts, and in support of this hypothesis we now propose to show that the so-called creatine of muscles is a post-mortem product and that there is very little creatine in living muscles.

Our reasoning is as follows: muscles of cats or rabbits yield 450-550 mgms. of creatine per 100 grams of fresh muscle. If this creatine exists as creatine in the muscles the muscles cannot absorb creatine from the circulating blood when the latter contains a very much smaller percentage of creatine than that indicated by the post-mortem analyses of the muscles. The fact that blood normally contains only traces of creatine, at most 8-10 mgms. per 100 grams, while the muscles yield 40-50 times as much shows clearly that there is some definite effective force or condition by which the creatine is held fast in the muscles. We know from feeding experiments that when creatine is fed to man or animals the total amount absorbed and kept by the animals is not very large. One gram of creatine per day is about the maximum amount which a full-grown man can be made to retain even when kept on low protein diets and on high protein diets the amounts retained are smaller than on diets low in protein. The fact that on feeding small amounts of creatine the greater part of it is retained indicates, however, that the tissues absorb it from the

<sup>8</sup> Nearly all the recent writers have in fact dodged the issue raised by Folin as to whether tissue creatine is still to be regarded as a waste product, though all who believe arginine to be a precursor of creatine (and creatinine) presumably regard creatine as a waste product.

blood—the apparently creatine-laden tissues have absorbed the minute amounts of creatine which circulate in the blood.

It may at once be granted that the validity of such indirect evidence of creatine absorption from the blood is not conclusive, though it is not easy to see how fed creatine can “disappear” except by getting into the tissues. Even the liver contains a larger concentration of tissue creatine than would occur in the blood as the result of such feeding experiments.

A temporary absorption of food creatine by tissues quite similar to the absorption of creatinine, urea, iodides, and other products which are either absent or present only in traces in the muscles, is tacitly admitted by all workers in the field, but the significance of such absorption in the case of creatine has not been recognized. Direct proof that a relatively small accumulation of creatine in the blood is accompanied by absorption of creatine by the muscles is furnished in experiments 6 and 7 of our third metabolism paper,<sup>9</sup> but at that time we were chiefly interested in the general phenomenon of absorption and urea formation, and therefore failed to grasp the significance of the results. Additional direct proof that living muscle contains very little free creatine is furnished by the absorption experiments recorded below.

These experiments were made in substantially the same way as our earlier absorption experiments.<sup>10</sup> In order to prevent loss of creatine (or creatinine) by way of the kidneys the blood supply of these organs was cut off by means of ligatures. After taking a preliminary sample of arterial blood and two samples of muscles (the gracilis for the total creatine determination and the adductor femoris and semimembranosus for the determination of the preformed creatinine) a freshly made solution of creatine was introduced in the small intestine. The latter was ligatured just below the stomach and just above the caecum. The creatine used was crystallized, *i.e.*, it contained its water of crystallization and was strictly pure. At the end of the absorption period samples of blood and muscle were taken for the second set of creatine (and creatinine) determinations. The analytical methods used in the determination of creatine and creatinine are described in the preceding papers in this number of the *Journal*.

EXPERIMENT 1. Cat 5 (weight 3650 grams) had fasted for twenty-four hours before the operation. After taking samples of

<sup>9</sup> This *Journal*, xii, p. 153, 1912.

<sup>10</sup> For a detailed description of the technique, see this *Journal*, xii, p. 144, 1912.

blood and muscle, 100 cc. of solution containing 3 grams of creatine were introduced into the small intestine.

	<i>mgms.</i>
1. Creatine per 100 grams of muscle before the injection...	544.0
2. Creatine per 100 grams of muscle 135 minutes after the injection.....	689.0
Creatine increase per 100 grams of muscle.....	145.0
3. Total creatine per 100 grams of blood before the injection	10.0
4. Total creatine per 100 grams of blood 130 minutes after injection.....	80.0
5. Preformed creatinine per 100 grams of blood before the injection.....	2.0
6. Preformed creatinine per 100 grams of blood 130 minutes after the injection.....	2.2
7. Preformed creatinine per 100 grams of muscle before the injection.....	8.0
8. Preformed creatinine per 100 grams of muscle 135 minutes after the injection.....	7.0

EXPERIMENT 2. Cat 6 (weight 3120 grams) had fasted twenty-four hours before the operation. After the usual preliminaries including the taking of samples of blood and muscle 100 cc. of solution containing 3 grams of creatine were introduced into the intestine. After two hours 1.6 grams of creatine were recovered from the intestine, showing that 1.4 grams of creatine had been absorbed.

	<i>mgms.</i>
1. Creatine per 100 grams muscle before the injection.....	475.0
2. Creatine per 100 grams muscle 120 minutes after the injection.....	558.0
Creatine increase per 100 grams of muscle.....	83.0
3. Total creatine per 100 grams of blood before the injection	8.0
4. Total creatine per 100 grams of blood 120 minutes after the injection.....	50.0
5. Preformed creatinine per 100 grams of blood before the injection.....	1.1
6. Preformed creatinine per 100 grams of blood 120 minutes after the injection.....	1.2
7. Preformed creatinine per 100 grams of muscle before the injection.....	6.6
8. Preformed creatinine per 100 grams of muscle 120 minutes after the injection.....	3.3

EXPERIMENT 3. Cat 7 (weight 2580 grams) had fasted for twenty-four hours before the operation. On account of the smaller

size of the animal only 2 grams of creatine dissolved in 75 cc. of water were injected into the intestine. The absorption was very extensive, corresponding to 1.66 grams in 135 minutes.

	<i>mgms.</i>
1. Creatine per 100 grams of muscle before the injection....	503.0
2. Creatine per 100 grams of muscle 135 minutes after the injection.....	565.0
Creatine increase per 100 grams muscle.....	62.0
3. Total creatine per 100 grams of blood before the injection	7.6
4. Total creatine per 100 grams of blood 135 minutes after the injection.....	122.0
5. Preformed creatinine per 100 grams of blood before the injection.....	1.1
6. Preformed creatinine per 100 grams of blood 135 minutes after the injection.....	1.2
7. Preformed creatinine per 100 grams of muscle before the injection.....	3.3
8. Preformed creatinine per 100 grams of blood 135 minutes after the injection.....	3.3

The three successive experiments recorded above prove conclusively that creatine is absorbed to an extraordinary extent by the muscles from the circulating blood. Indeed in the first two experiments the accumulation of creatine in the muscles is greater than the accumulation in the blood. We are not now prepared to offer an explanation for this fact but intend to make it the subject of further investigation.<sup>11</sup> As an additional demonstration of the rapidity with which the muscles absorb creatine we have also injected moderate quantities of creatine directly into the blood (jugular vein). The results of one such experiment are recorded below.

EXPERIMENT 4. Cat 8 (weight 4550 grams) had fasted twenty-four hours before the operation. After taking samples of blood and muscles and shutting off the kidneys by ligatures we injected in the course of thirty-two minutes 50 cc. of creatine solution containing 0.66 gram of creatine.

<sup>11</sup> The question whether the creatine of meat and meat extracts is a valuable food ingredient is not affected by the interpretation of tissue creatine as a constituent of the protoplasm.

	<i>mgms.</i>
1. Creatine per 100 grams muscle before the injection.....	498.0
2. Creatine per 100 grams muscle 60 minutes after the end of the injection.....	544.0
Creatine increase per 100 grams of muscle.....	46.0
3. Total creatine per 100 grams of blood before the injection	6.3
4. Total creatine per 100 grams of blood 5 minutes after injection.....	50.0
5. Total creatine per 100 grams of blood 60 minutes after the injection.....	43.0
6. Preformed creatinine per 100 grams of blood before the injection.....	1.4
7. Preformed creatinine per 100 grams of blood 5 minutes after the injection.....	2.1
8. Preformed creatinine per 100 grams of blood 60 minutes after the injection.....	1.9
9. Preformed creatinine per 100 grams of muscle before the injection.....	2.3
10. Preformed creatinine per 100 grams of muscle 60 minutes after the injection.....	3.4

Five minutes after the injection the distribution of the administered creatine between the blood and the muscles was evidently almost completed since the creatine content of the blood fell only from 50 mgms. to 43 mgms. in the course of the following fifty-five minutes.

These results warrant, we believe, the conclusion that the absorption of creatine from the blood by living muscles is just as rapid and extensive (if not more so) than the corresponding absorption of urea, creatinine or amino-acids. Our explanation of this phenomenon as already stated is that living muscles contain virtually no creatine, and that the creatine found on analysis is a post-mortem product originally constituting a part of the living protoplasm.

It is conceivable of course that creatine is present in muscles in some other form of organic combination than that with the living material, but the organic compound involved is an extraordinarily unstable one since the creatine is set free when muscles (of frogs) are killed by mechanical injury so that it can then be extracted with water or saline solutions.<sup>12</sup>

<sup>12</sup> Mr. T. E. Buckman in this laboratory is now investigating this subject from a quantitative standpoint.

But the one important striking change in muscles produced by mechanical injury is the transformation of living protoplasm into residues that are dead. It seems reasonable to assume that corresponding to such a momentous biological change important chemical transformations must take place, and we need not assume that the chemical transformations accompanying the death of protoplasm consist of obscure intramolecular rearrangements which cannot be revealed by chemical analysis. As we look at it now the protein obtained from protoplasm represents but one group of substances into which the living materials split up when the characteristic vital phenomena disappear. Other substances, among which creatine is one in the case of vertebrate tissues, must be equally important for an adequate understanding of the chemical architecture of the intact protoplasmic units. We have no desire to enlarge upon this fascinating aspect of the subject. We refer to it only because if the line of reasoning underlying the above experiments and conclusions is sound then we have before us a perfectly definite experimental procedure by means of which other constituents of the protoplasm of higher animals may be identified as such. All that is necessary for such investigations is to find suitable analytical methods permitting the determination in blood and tissues of a suspected protoplasmic component, and then determine, as we have done with creatine, whether the substance under investigation is absorbed from the blood into regions of apparently higher concentration.

The hypothesis that creatine is a part of living vertebrate protoplasm would seem to permit a more plausible explanation of the occurrence and behavior of creatine and creatinine in relation to metabolism than any heretofore advanced. 1. When a tissue dies the post-mortem product creatine is set free, whereas in the course of the normal, replaceable breakdown which we call tissue metabolism the product split off is usually not creatine but creatinine. 2. In times of unusual stress such as in fevers, sometimes in fasting, and in various pathological conditions, the normal breakdown into creatinine is accompanied by more or less abnormal breakdown into creatine. 3. Traces of creatine in the urine might occur as a result of its spontaneous formation from the original catabolism product creatinine, just as traces of creatinine (but no more) may be produced out of creatine taken with the

food. 4. The various hypothetical ferments of Gottlieb and Stanggasser<sup>13</sup> for the transformation of creatine and creatinine into one another, as well as for their destruction, in so far as they have any existence at all, represent post-mortem conditions, and are superfluous according to this point of view of the metabolism of creatine and creatinine. 5. The reason why it has been found impossible to trace the formation of creatine and creatinine to any constituent of the food is that it is not possible to increase the mass of the tissues by feeding, for, according to the point of view here presented, creatine is synthesized only in connection with the growth or renewal of the protoplasm. 6. The creatinine elimination becomes more clearly than ever the most clear cut index or measure of the total normal tissue metabolism. 7. The liver has no special function to perform in connection with the creatinine formation.

Why creatine should occur in the urine of children, and presumably in the urine of other young growing animals is not yet clear. We need more extensive analyses of the blood and tissues of such animals before it becomes worth while to try to explain this phenomenon.

The alleged excretion of creatine instead of creatinine by animals in whose metabolism uric acid largely takes the place of urea as the chief nitrogenous waste product also remains obscure. The observations of Meissner,<sup>14</sup> Paton,<sup>15</sup> Voegtlin, etc., as to the facts in the case of birds stand in need of further study.

The creatinine figures recorded in connection with our experiments clearly indicate that the creatinine does originate in the muscles, since the preformed creatinine found in the muscles, small as it is, is nevertheless invariably greater than the preformed creatinine found in the blood. We have several other experiments in addition to those recorded in this paper all of which have yielded the same result.

Another interesting fact which we have discovered in the course of these investigations is that though the heart yields very much less creatine than do the striated muscles—yet in nearly every case the heart contains more preformed creatinine than is found in the striated muscles. This is true

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<sup>13</sup> *Zeitschr. f. physiol. Chem.*, lii, p. 1, 1907; lv, p. 322, 1908; Mellanby: *Journ. of Physiol.*, xxxvi, p. 447, 1908.

<sup>14</sup> *Zeitschr. f. rationelle Medizin*, xxiv, p. 297, 1868.

<sup>15</sup> *Loc. cit.*

in the case of rabbits as well as cats. In the striated muscles of rabbits there are about 3 mgms. of creatinine per 100 grams, while in the heart we find 4 mgms. or more. These observations do not agree very well with the hypothesis of Myers and Fine according to which the creatinine formation should represent a definite proportion of the tissue creatine. According to our point of view the figures simply indicate that the tissue metabolism in the heart is very much more rapid than the tissue metabolism in the voluntary muscles.

Our experiments have failed to show any creatinine formation out of the administered creatine. There is a slight accumulation of creatinine in the blood and a slight diminution of the creatinine in the muscles. It looks as if the unusually large influx of creatine into the muscles tended to force out the creatinine, but the variations are so small that we hesitate to attach much significance to them although we have observed the phenomenon many times. If correct, this observation will help to explain the findings of those investigators who believe that the administration of creatine is accompanied by a demonstrable increase in the creatinine elimination.

STUDIES ON THE SYNTHESIS OF HIPPURIC ACID IN  
THE ANIMAL ORGANISM. I. THE SYNTHESIS  
OF HIPPURIC ACID IN RABBITS ON A  
GLYCOCOLL-FREE DIET.

By HOWARD B. LEWIS.

(From the Department of Physiological Chemistry of the University of  
Pennsylvania, Philadelphia, Pa.)

(Received for publication, March 18, 1914.)

The problem of the production of glycocoll in the animal organism for the synthesis of hippuric acid after the ingestion of benzoates has been studied by many investigators with varying results. Of the older investigators, Wiechowski<sup>1</sup> maintained that the glycocoll content of the tissues is insufficient to account for the amount of glycocoll eliminated in combination with benzoic acid and that glycocoll can be synthesized at the expense of the urea appearing in metabolism. More recently Ringer<sup>2</sup> observed an increased nitrogen elimination greater than the amount of nitrogen eliminated as hippuric acid. He suggests that the large quantities of glycocoll originate from the "extra destroyed protein" and not from the protein that would have been metabolized had no benzoic acid been given, *i.e.*, glycocoll results from a specific metabolism, not from a deviation of normal metabolism. Epstein and Bookman<sup>3</sup> conclude that the production of hippuric acid results from a selective process which results in the elimination of large amounts of nitrogen, largely in the form of hippuric acid nitrogen. In both of these investigations the urea plus ammonia nitrogen was found to remain unaltered on the day of the benzoic acid ingestion. Recently McCollum and Hoagland<sup>4</sup> employing pigs reduced to their minimum level of endogenous nitrogenous

<sup>1</sup> Wiechowski: *Beitr. z. chem. Physiol.*, vii, p. 204, 1905.

<sup>2</sup> Ringer: *this Journal*, x, pp. 327-38, 1911.

<sup>3</sup> Epstein and Bookman: *ibid.*, x, p. 353, 1911.

<sup>4</sup> McCollum and Hoagland: *ibid.*, xvi, p. 321, 1913.

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metabolism on a protein-free diet of ample calorific value found that with amounts of benzoic acid not excessive there was no noticeable rise in the elimination of nitrogen and that much of the nitrogen normally appearing in the urine as urea nitrogen may be eliminated as hippuric acid nitrogen. They suggest that the discrepancy between their results and those of previous investigators may be due to the shorter periods of observation where temporary rises in nitrogenous metabolism may obscure the fall in the urea nitrogen, or to the fact that the animals used by others (goat, rabbits, etc.) may have another type of metabolism.

In view of the results of McCollum and Hoagland, it seemed of interest to investigate the urea elimination after the feeding of benzoic acid, since the work of others had shown that in rabbits no influence on the urea elimination was to be observed. The rabbits received a diet sufficient in protein content, but containing no glycocholate. To accomplish this the animals were maintained on a constant milk ration, a diet which for practical purposes may be regarded as glycocholate-free, since the main protein of milk, casein, contains no glycocholate. The observation made by Laqueur<sup>5</sup> that rabbits can be kept over long periods of time on a diet of milk alone has greatly simplified the maintenance of these animals on a constant diet for metabolism studies. The ease of exact analysis of the diet, the possibility of a constant input of liquid and food (since the diet may be administered quantitatively through a stomach tube, if the animal refuses to eat) make the method extremely valuable in work with rabbits and other similar small animals. In one experiment (Rabbit E) the animal was kept on this milk diet for a period of seventeen days without loss of weight. No disorders of the alimentary tract were encountered.

The urine was collected by emptying the bladder by gentle pressure daily at the same hour. The milk fed was divided into two portions and administered through a stomach tube at 9 a.m. and 3 p.m. daily. When the benzoate was to be added to the diet, half of the total amount of the benzoate was given with each portion of milk. Benedict's method was used for the determination of urea, and the method of Folin and Flanders for the determination of hippuric acid. The latter method gives figures which repre-

<sup>5</sup> Laqueur: *Zeitschr. f. physiol. Chem.*, lxxxiv, p. 109, 1913.

sent the total benzoic acid of the urine, but since in rabbits the benzoic acid excreted free, or conjugated, other than as hippuric acid is slight, the figures may be taken as representing the hippuric acid elimination with but slight error. As the elimination of ammonia nitrogen by the rabbit is so small, no separate determination of it was made.

In confirmation of the work of McCollum and Hoagland, there was observed a marked decrease in the urea + ammonia nitrogen output after the feeding of sodium benzoate as compared with the control periods, a decrease which is almost entirely accounted for by the nitrogen eliminated as hippuric acid. This decrease was especially marked in the experiments with Rabbits E and H, in which larger doses of sodium benzoate were administered. Thus in the case of Rabbit E after the administration of 3 grams of the benzoate, the urea + ammonia nitrogen fell from an average of 0.765 gram in the fore period (85.7 per cent of the total nitrogen eliminated) to 0.431 gram on the experimental day (54.8 per cent of the total nitrogen). Similarly in the experiments on Rabbit H, the urea + ammonia nitrogen figures changed from an average of 0.765 gram to 0.481 gram on the experimental day. There was no notable increase in the elimination of total nitrogen due to excessive breakdown of body tissue as a result of the benzoate ingestion, even in the experiments on Rabbits E and H in which doses of 3 grams of sodium benzoate, nearly the lethal dose for animals of this weight, were given. If the nitrogen present as hippuric acid be subtracted from the undetermined nitrogen, the figures obtained (shown in the tables in parentheses) are quite comparable to those representing the undetermined nitrogen of the control period, *i.e.*, there is no increase in undetermined nitrogen indicative of a specialized catabolism of protein as a source of glyocoll.

Since the diet contained no glyocoll, the glyocoll eliminated as hippuric acid must originate from one of two sources, either from preformed glyocoll of the tissues or from the amino-acids present in the diet or in the cell proteins normally catabolized. Inasmuch as no increased nitrogen elimination sufficient to account for a breakdown of enough tissue protein to yield the required amount of preformed glyocoll was observed, we would seem to be justified in assuming that part of the nitrogen normally eliminated as urea may function in the synthesis of hippuric acid. Whether this

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DATE	VOLUME cc.	TOTAL N gram	UREA + NH <sub>3</sub> N gram	UREA + NH <sub>3</sub> N per cent	UNDETER- MINED N gram	HIPPURIC ACID grams	HIPPURIC ACID N gram	N IN DIET grams	DIET
<i>Rabbit A. Weight 1920 grams.</i>									
1913 Dec. 8	125	1.044	0.883	84.6	0.161			1.10	200 cc. milk.
9	205	1.085	0.691	63.7	0.394 (0.225)	2.16	0.169	1.10	{ 200 cc. milk + 2 gms. sodium benzoate.
10	105	0.908	0.724	79.7	0.184			1.10	200 cc. milk.
11	100	0.814	0.637	78.2	0.177			1.10	200 cc. milk.
12	170	0.904	0.724	82.3	0.160			1.10	200 cc. milk.
<i>Rabbit C. Weight 1300 grams.</i>									
Dec. 15	130	0.701	0.584	83.3	0.116			0.833	150 cc. milk.
16	115	0.830	0.727	87.6	0.103			0.806	150 cc. milk.
17	130	0.720	0.620	86.1	0.100			0.806	150 cc. milk.
18	155	0.804	0.546	67.9	0.258 (0.096)	2.07	0.162	0.822	{ 150 cc. milk + 2 gms. sodium benzoate.
19	135	0.691	0.606	87.7	0.085			0.822	150 cc. milk.
20	115	0.624	0.533	85.4	0.091			0.822	150 cc. milk.
<i>Rabbit D. Weight 1520 grams.</i>									
1914 Jan. 12	120	0.734	0.625	85.3	0.109			1.10	200 cc. milk.
13	130	0.594	0.524	88.2	0.070			1.10	200 cc. milk.
14	185	0.721	0.454	63.0	0.267			1.10	{ 200 cc. milk + 2 gms. sodium benzoate.
15	140	0.827	0.710	85.8	0.117			1.10	200 cc. milk.
16	100	0.802	0.668	83.3	0.134			1.10	200 cc. milk.
17	125	0.832	0.704	84.6	0.128			1.10	200 cc. milk.

## Rabbit E.

DATE	WEIGHT grams	VOLUME cc.	TOTAL N gram	UREA + NH <sub>2</sub> N gram	UREA + NH <sub>2</sub> N per cent	UNDETER- MINED gram	HIPPURIC ACID grams	HIPPURIC ACID N gram	N IN DIET grams	DIET
1914										
Jan. 7	1710	150	1.047	0.918	87.7	0.129			1.10	200 cc. milk.
8	1720	165	0.943	0.828	87.8	0.115			1.10	200 cc. milk.
9	1720	155	1.026	0.890	86.6	0.136			1.10	200 cc. milk.
10	1720	170	1.143	0.825	72.2	0.318 (0.141)	2.26	0.177	1.10	{ 200 cc. milk + 2 gms. sodium benzoate.
11	1720	150	0.886	0.731	82.5	0.155			1.10	200 cc. milk.
12	1710	135	0.842	0.725	86.1	0.117			1.10	200 cc. milk.
16	1720	145	0.878	0.757	86.4	0.121			1.10	200 cc. milk.
17	1720	150	0.908	0.772	85.0	0.126			1.10	200 cc. milk.
18	1720	190	0.787	0.431	54.8	0.356 (0.120)	3.02	0.236	1.10	{ 200 cc. milk + 3 gms. sodium benzoate.
19	1650	130	0.977	0.814	83.3	0.163			1.10	200 cc. milk.
20	1700	155	0.948	0.787	83.0	0.161			1.10	200 cc. milk.

## Rabbit H.

DATE	WEIGHT grams	VOLUME cc.	TOTAL N gram	UREA + NH <sub>2</sub> N gram	UREA + NH <sub>2</sub> N per cent	UNDETER- MINED gram	HIPPURIC ACID grams	HIPPURIC ACID N gram	N IN DIET grams	DIET
Mar. 10	2070	95	0.940	0.819	87.0	0.121			1.10	200 cc. milk.
11	2050	140	0.808	0.720	89.1	0.088			1.10	200 cc. milk.
12	2050	155	0.878	0.757	86.2	0.121			1.10	200 cc. milk.
13	2040	195	0.863	0.481	55.7	0.382 (0.126)	3.275	0.256	1.10	{ 200 cc. milk + 3 gms. sodium benzoate.
14	2000	100	0.795	0.655	82.4	0.140			1.10	200 cc. milk.
15	2020	115	0.756	0.631	83.4	0.125			1.10	200 cc. milk.

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deviation of nitrogen from its normal path of metabolism results from a building down of higher amino-acids to glycocoll before deaminization, or from a synthesis *de novo* of glycocoll from ammonia and a fatty acid derivative cannot be stated. Further investigations of this problem are being carried on.

### SUMMARY.

In rabbits fed on a glycocoll-free diet of milk, the ingestion of sodium benzoate causes no marked rise in the elimination of total nitrogen. The nitrogen eliminated as hippuric acid appears to be derived at the expense of the nitrogen normally present in the urine as urea, since the urea elimination decreases with increased hippuric acid elimination. The synthesis of glycocoll for the purposes of detoxication of the benzoate results from a deviation of the normal path of catabolism and not from a specialized metabolism. These results are in agreement with those obtained by McCollum and Hoagland in their studies on pigs reduced to their endogenous protein metabolism.

# THE CONSTITUTION OF KYNURENIC ACID.

By ANNIE HOMER.

(From the Physiological Laboratory, Cambridge, the Lister Institute, London, and the Biochemical Institute, University of Toronto.)

(Received for publication, March 20, 1914.)

## HISTORICAL.

Kynurenic acid was first isolated from the urine of dogs by Liebig<sup>1</sup> (1853) but owing to the scarcity of material at his disposal he did not make any attempt to ascertain the constitution of the substance. In 1858<sup>2</sup> he fed dogs on meat for several weeks and was able to obtain sufficient of the substance for analysis. Liebig assigned the formula  $C_{16}H_7NO_6$  to kynurenic acid.

Schneider<sup>3</sup> analyzed the acid and suggested for it the formula  $C_{20}H_9NO_6$ . Schmiedeberg and Schultzen<sup>4</sup> (1872) analyzing the free acid and its barium salt obtained results in accordance with the formula  $C_{20}H_{14}N_2O_6 \cdot 2H_2O$  for the acid. They demonstrated that at a temperature of 150° the acid became anhydrous; that on heating the acid to the temperature at which it melts carbon dioxide was evolved and a substance which they called kynurin (m.p. 201°) was formed. Kynurin and its platinum salt on analysis gave results in accordance with the formula  $C_{18}H_{14}N_2O_2$  for the former.

Kretschy (1881, 1883, 1884),<sup>5</sup> assigned the formula  $C_{10}H_7NO_3 \cdot H_2O$  to the acid and made the following observations with regard to kynurin and kynurenic acid:

1. Kynurin is of a phenolic nature.
2. Kynurin on distillation with zinc dust yields quinoline.
3. Kynurenic acid on distillation with hydrochloric acid and zinc dust yields quinoline.
4. Kynurenic acid on oxidation with alkaline permanganate is converted into oxalic acid and an acid to which he gave the name kynuric acid.
5. Kynuric acid on hydrolysis with water gave oxalic acid and *o*-amido benzoic acid: on treatment with potash and potassium carbonate aniline was formed.

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<sup>1</sup> Liebig's *Annalen*, lxxxvi, p. 125.

<sup>2</sup> *Ibid.*, cviii, p. 354.

<sup>3</sup> Schneider: *Sitz. d. Wien. Akad. d. Wissensch.*, lix, p. 24.

<sup>4</sup> Schmiedeberg and Schultzen: *Annalen*, clxiv, p. 155.

<sup>5</sup> Kretschy: *Monatsh. f. Chem.*, v, p. 16; iv, p. 156; *Wien. Akad. Ber.*, lxxxiii, 2, p. 171.

Analysis of kynuric acid gave results agreeing with the formula  $C_9H_7NO_4$ .

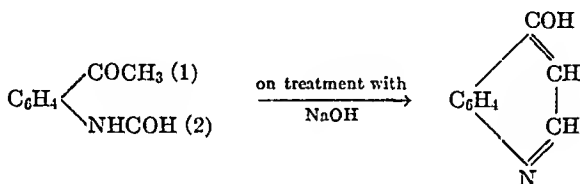
From the above observations it is clear that kynuric acid has the structure  $C_6H_4(COOH)(NHCO \cdot COOH)$  and that kynurenic acid is a hydroxy-

quinoline carboxylic acid.

The melting point of kynurenic acid has been stated by Schmiedeberg and Schultzen as  $264-266^\circ C$ . and by Kretschy as  $257-258^\circ C$ . Liebig does not mention the melting point of his specimens of the acid.

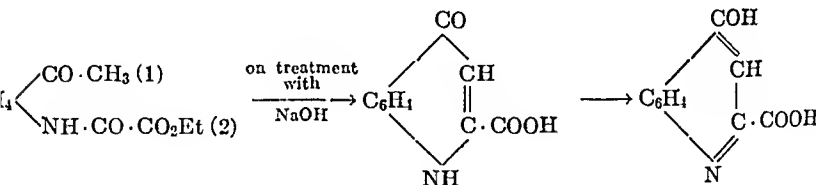
Wenzel<sup>6</sup> prepared kynurin from cinchoninic acid and showed that it was  $\gamma$ -hydroxy-quinoline.

Camps<sup>7</sup> (1901) in order to ascertain the constitution of kynurenic acid synthesized kynurin from formyl-*o*-amidoacetophenone thus furnishing further evidence that the HO group is in the  $\gamma$  position.

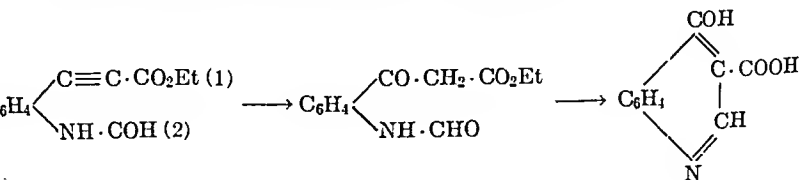


He also synthesized two hydroxy-quinoline carboxylic acids:

(1)  $\gamma$ -Hydroxy- $\alpha$ -carboxy-quinoline by the action of caustic soda on the anilide formed from oxalic ester and *o*-amido-acetophenone thus:



and (2)  $\gamma$ -hydroxy- $\beta$ -carboxy-quinoline by the action of caustic soda on the formyl anilide of *o*-amidophenyl propiolic ester.



<sup>6</sup> Wenzel: *Monatsh. f. Chem.*, xv, p. 453, 1894.

<sup>7</sup> Camps: *Zeitschr. f. physiol. Chem.*, xxxiii, p. 390.

Camps states that the  $\gamma, \beta$  derivative melts at 266–267°C, and the  $\gamma, \alpha$  derivative at 290°C. and comments on the different values for the melting point of the natural acid given by Schmiedeberg and Schultzen and by Kretschy. Accepting the value 264–266° given by the former he concludes that Liebig's kynurenic acid must be the  $\gamma$ -hydroxy- $\beta$ -carboxy derivative. He does not mention having handled any of the natural product for himself or having attempted to verify his conclusions by the simple experiment of taking the melting point of a mixture of the natural with each of the synthetic products.

Since kynurenic acid was first isolated by Liebig much work has been done by various experimenters<sup>8</sup> to ascertain the significance of this substance in the metabolic processes of the animal body. Finally Ellinger<sup>9</sup> (1904) showed that its precursor is tryptophane. He administered doses of tryptophane to dogs and estimated the increased output of kynurenic acid. The latter substance was identified by its melting point, its reactions and by the percentage of barium in its barium salt. Ellinger does not mention the melting point of the acid but states that the substance although pigmented melts at the right temperature.

The conversion of tryptophane into kynurenic acid involves the introduction of an extra carbon atom into the indole ring. How such a remarkable type of chemical reaction can take place in the animal body is a problem of great interest the theoretical aspect of which will be fully discussed in a later paper.

#### EXPERIMENTAL.

During the course of some metabolism experiments I noticed that the kynurenic acid isolated from the urine of dogs which had been kept on a meat diet or to which tryptophane had been administered, although deeply pigmented and contaminated with uric acid, could be purified as follows:

The brown colored substance obtained from urine was dissolved in ammonia, the solution was made slightly acid with acetic acid and allowed to stand for twenty-four hours during which time a brown amorphous precipitate was slowly deposited. After filtration from this pigmented substance the clear filtrate on further acidification with 4 per cent hydrochloric acid gave an almost colorless precipitate which melted at 278°. Previous to the removal of the brown precipitate the acid had not melted sharply but had become sticky at about 260°. The kynurenic acid at this stage was usually contaminated with a small amount of uric acid and in order to get rid of this impurity the precipitated acid

<sup>8</sup> Voit and Reidener, Hauser, Nigeller, Mendel, Giacosa, Bauman, Solomni and others.

<sup>9</sup> Ellinger: *Zeitschr. f. physiol. Chem.*, xliii, p. 325.

was again dissolved in ammonia and the uric acid precipitated by Hopkin's method (*i.e.*, by saturation with ammonium chloride). After the removal of the uric acid by filtration the filtrate was acidified with 4 per cent hydrochloric acid and the kynurenic acid thus thrown down was filtered off, washed and dried. It was further purified by two crystallizations from alcohol in which it is soluble to the extent of 0.1 gram in 100 cc. of the solvent. It was finally crystallized from 40 per cent acetic acid until a constant melting point was obtained. The acid melted, with evolution of carbon dioxide, at 288–289° (uncorr.).

Samples of kynurenic acid were prepared from the urine of five different dogs which had been fed on meat alone, on meat and doses of tryptophane and on bread and milk and tryptophane, and in every case the melting point of the purified acid was 288–289° sharp. Another sample of the acid which Mr. Laidlaw of Guy's Hospital, London, kindly sent me, after the above described treatment, also melted at 289°.

The natural acid thus melts at a temperature 22° higher than the value given by Schultzen and accepted by Camps and used by him to decide the question as to which of the hydroxy-quinoline acids kynurenic acid is.

That the acid isolated in the above experiments is an hydroxy-quinoline acid is demonstrated by the following observations: On heating the substance with zinc dust a smell of quinoline is noticed, the acid gives Jaffé's color reaction for  $\gamma$ -hydroxy-quinolines,<sup>10</sup> it gives Brieger's bromine test<sup>11</sup> and on being heated for some time at its melting point it loses CO<sub>2</sub> with the formation of a substance which after repeated crystallization from alcohol melts at 202°. One of Camps' synthetic hydroxy-quinoline acids (post P.) was taken and under the same conditions gave a crystalline derivative which melted at 202°. Admixture of this substance with the derivative obtained similarly from natural kynurenic acid caused no change in the melting point, thus proving the identity of the two products. Camps and Wenzel have shown that the substance thus formed and known as kynurin is  $\gamma$ -hydroxy-quinoline. (Camps, Schmiedeberg and Schultzen and Kretschy give the melting point of kynurin as 201°.)

<sup>10</sup> Jaffé: *Zeitschr. f. physiol. Chem.*, vii, p. 399.

<sup>11</sup> Brieger: *Ibid.*, iv, p. 89.

It is clear from a consideration of the melting point of the samples of kynurenic acid handled by me that the natural product (m. p. 289°) cannot have the constitution of Camps'  $\gamma$ -hydroxy- $\beta$ -carboxy-quinoline (m. p. 265–266°) but there remained the alternative that the acid, since it is a quinoline acid, might be the isomeric  $\gamma$ -hydroxy- $\beta$ -carboxy-quinoline (m. p. 290°). In order to test this possibility I repeated Camps' synthesis of  $\gamma$ -hydroxy- $\alpha$ -carboxy and of  $\gamma$ -hydroxy- $\beta$ -carboxy-quinolines.

### 1. $\gamma$ -Hydroxy- $\alpha$ -carboxy-quinoline.

Camps' synthesis was followed in detail. The anilido compound formed by the action of oxalic ester on *o*-amidoacetophenone was prepared and after recrystallization from alcohol melted at 128° (Camps states m. p. 128°). The anilide compound on hydrolysis with caustic soda and subsequent acidification yielded an acid which can only have the  $\gamma$ -hydroxy- $\alpha$ -carboxy constitution (p. 510).

The following comparison establishes the identity of this  $\gamma$ -OH,  $\alpha$ -COOH quinoline with the natural acid. The substances were dried at 150°C. for analysis.

0.1060 gram synthetic acid gave 0.2450 gram CO<sub>2</sub> and 0.0370 gram H<sub>2</sub>O.

0.1644 gram synthetic acid gave 9.9 cc. moist N<sub>2</sub> at 13.25°, 774.3 mm.

0.1012 gram natural kynurenic acid gave 0.2361 gram CO<sub>2</sub> and 0.0370 gram H<sub>2</sub>O.

0.1628 gram natural kynurenic acid gave 10.2 cc. moist N<sub>2</sub> at 14°, 769 mm.

	CAMPS' SYNTHETIC ACID PREPARED BY		NATURAL KYNURENIC ACID PURIFIED AS ABOVE	THEORY FOR C <sub>10</sub> H <sub>7</sub> NO <sub>2</sub>
	Camps (1901)	Homer		
C.....	63.39	63.04	63.62	63.49
H.....	3.82	3.88	4.06	3.70
N.....	7.52	7.29	7.49	7.41
M.P.....	290°	288–289°	288–289°	

A mixture of Camps' acid and the natural product melted at 288–289° (uncorr.) thus proving the identity of the two compounds.

Natural kynurenic acid must therefore be regarded as  $\gamma$ -hydroxy- $\alpha$ -carboxy-quinoline and not as the  $\gamma$ ,  $\beta$  isomer as hitherto stated.

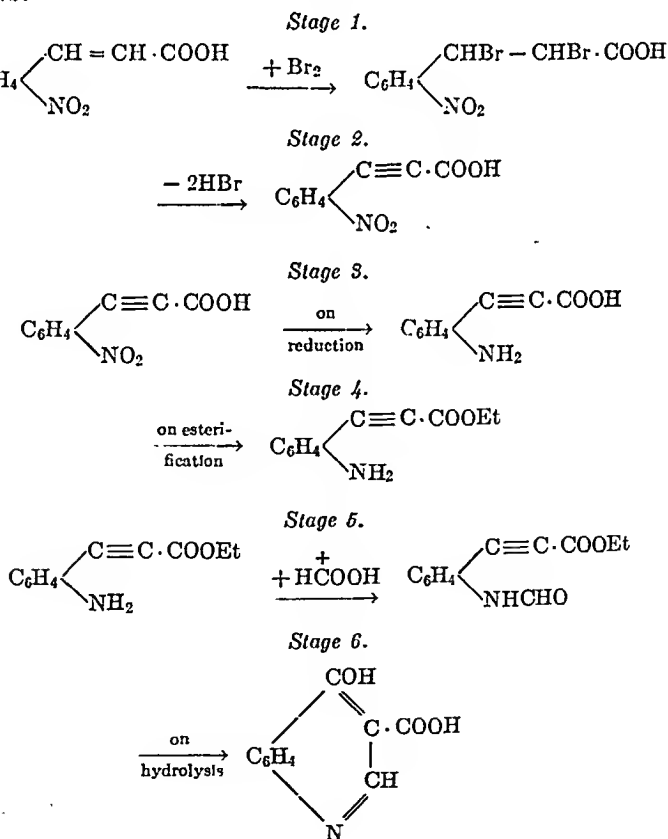
In spite of the conclusive evidence as to the constitution of the

natural acid it seemed advisable, in order to make the work more complete, to repeat the work of Camps on the synthesis of the isomeric quinoline acid (on p. 266-267) and to compare the properties of this substance with those of the natural acid.

## 2. Synthesis of $\gamma$ -hydroxy- $\beta$ -carboxy quinoline.

Camps' directions were followed in detail but the synthesis proved difficult to accomplish. Six attempts which entailed work extending over a period of twelve months were made. The products at each stage were analyzed in order to ascertain at which point the synthesis had failed in the author's hands.

The stages of the process may be represented graphically as follows:



It was found that the formation of the formyl derivative (stage 5) did not proceed satisfactorily. In the first three attempts it transpired that there had been no formation of this derivative when *o*-amidophenylpropionic ester and formic acid were left together in solution in specially dried ether at 0°C. for periods of twenty-four hours. Camps states that this reaction proceeds quantitatively under these conditions. In a fourth experiment the temperature of the reaction was raised but without better result. In the fifth and sixth attempts the reaction mixture was kept at 0°C. for five days and at 2°C. for a further period of two days. In both cases a small amount of formyl derivative was obtained which after hydrolysis with soda and subsequent acidification gave rise to the formation of a crystalline acid. The identity of the end product in both cases was established.

That the acid thus synthesized was a quinoline acid was demonstrated by the following tests: it gave Jaffé's color reaction for  $\gamma$ -hydroxy-quinolines, it gave Brieger's bromine test and on being heated with zinc dust a smell of quinoline was evolved.

Admixture of this acid with Camps'  $\gamma$ -hydroxy- $\alpha$ -carboxy-quinoline and also with natural kynurenic acid caused a depression of their respective melting points.

Analysis of the acid which has been twice precipitated from its solution in ammonia well washed with water, and then recrystallized twice from alcohol and dried at 120° C. gave the following:

0.1135 gram gave 0.2427 gram CO<sub>2</sub> and 0.0439 grams H<sub>2</sub>O.

0.1123 gram gave 0.2397 gram CO<sub>2</sub> and 0.0414 gram H<sub>2</sub>O.

0.1330 gram gave 8 cc. of moist N at 18° and 760.4 pressure.

	Calculated for C <sub>10</sub> H <sub>7</sub> NO <sub>2</sub> .H <sub>2</sub> O:	Found:	
C.....	58.0	58.1	58.2
H.....	4.34	4.3	4.0
N.....	6.76	6.97	

The acid contained no halogen.

According to Camps the  $\gamma$ -hydroxy- $\beta$ -carboxy-quinoline obtained by him melted at 266–267°C.: its identity was established by the Jaffé reaction and by its decomposition into kynurin.

The acid obtained by me in these experiments and used for analysis although colorless had no sharp melting point: further recrystallization of both specimens was without effect on the

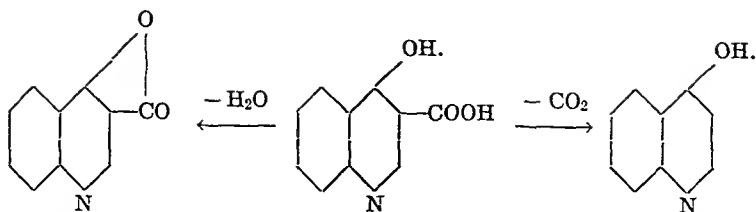
melting point. It was found that the acid became viscous at about 235°C. and was not completely fused until a temperature of 265°C. had been reached. On heating the substance to a temperature of about 270°C. it was noticed that a sublimate collected on the sides of the tube. The formation of the sublimate was not accompanied by the evolution of CO<sub>2</sub>. The sublimate after repeated crystallization from alcohol was found to melt at 250°C. sharp. A mixture of this and the original acid melted at 235°C. Both samples of the synthesized acid yielded the same sublimate on fusion.

The behavior of the synthesized acid towards heat points to the formation of an internal anhydride thus causing the indefinite melting point. Unfortunately after the above analysis there was very little of the synthesized acid left for further experiments and in consequence it was impossible to obtain enough of the sublimate for a quantitative demonstration of the formation of an anhydride. But the following experiment furnishes evidence of a qualitative nature in favor of this assumption, although the author does not wish to lay too great stress on evidence of this nature.

The original acid gave Jaffé's color reaction, the sublimate does not. The sublimate is insoluble in water and in 10 per cent caustic soda unless the temperature of the latter be raised. If the alkaline solution of the acid be heated for a short time and evaporated to dryness on a water bath the residue will then give the color reaction for  $\gamma$ -hydroxy-quinolines.

It was further noticed that if the synthetic acid be more strongly heated, *i.e.*, to a temperature considerably higher than the melting point, there is evolution of CO<sub>2</sub> gas and the formation of a sublimate which after being resublimed and then recrystallized once from alcohol melted at 202°C. (This substance gave the Jaffé color reaction and it is probably  $\gamma$ -hydroxy-quinoline, although owing to scarcity of material its identity with kynurin could not be established.)

It is probable that the hydroxy-quinoline acid formed from *o*-nitrophenylpropionic acid is decomposed by heat into two different substances according to the temperature employed, thus:



Whether the hydroxy-quinoline acid which the author has isolated is the same as that obtained by Camps cannot be stated at present. All that can be definitely said is that a repetition of the synthesis of  $\gamma$ -hydroxy- $\beta$ -carboxy-quinoline has resulted in the formation of a quinoline carboxylic acid giving the color test for  $\gamma$ -hydroxy-quinolines. The acid on gentle fusion yields a compound m. p.  $250^{\circ}\text{C}$ . which no longer gives the Jaffé color reaction but which will do so if hydrolyzed with soda. If the acid be more strongly heated there is loss of  $\text{CO}_2$  and a substance is formed which melts at  $202^{\circ}$  (uncorr.) and gives Jaffé's color reaction; this latter substance is probably  $\gamma$ -hydroxy-quinoline. Further the acid is different from natural kynurenic acid and from Camps'  $\gamma$ -hydroxy-carboxy-quinoline.

The author of this paper is unwilling to leave the investigation in its present state but the question as to whether the compound under investigation is the same as that isolated by Camps as  $\gamma$ -hydroxy- $\beta$ -carboxy-quinoline is a problem of a purely chemical nature, the elucidation of which would take a considerable time and must be temporarily postponed for the reason that the work described in this paper was primarily undertaken with a view to settling the constitution of natural kynurenic acid.

Between kynurenic acid and tryptophane there is some close though obscure connection in the metabolic functions of the dog. A long series of experiments has been undertaken in conjunction with Dr. F. Gowland Hopkins to ascertain how the former is produced in the animal body, but before making any speculations on this matter it is essential that the constitution of the acid be satisfactorily settled. The evidence adduced in the earlier part of this paper (p. 513) shows that the natural acid is identical with Camps'  $\gamma$ -hydroxy- $\alpha$ -carboxy-quinoline.

## SUMMARY.

1. Natural kynurenic acid when pure melts at 289°C. (uncorr.).

2. The identity of the natural acid with Camps' synthetic  $\gamma$ -hydroxy- $\alpha$ -carboxy-quinoline has been established.

Kynurenic acid has been hitherto regarded as  $\gamma$ -hydroxy- $\beta$ -carboxy-quinoline and its melting point has been given as 257-258° and 266-267°.

3. Attempts to synthesize Camps'  $\gamma$ -hydroxy- $\beta$ -carboxy-quinoline have been made. An acid has been isolated which gives reactions for a  $\gamma$ -hydroxy-quinoline acid. It differs from natural kynurenic acid but whether it is the same acid as that claimed to have been isolated by Camps cannot be stated with any degree of certainty until a complete chemical investigation of the reaction has been made. For the purpose of the present investigation the delay which such work would entail is inadvisable as it is of great importance to publish the positive evidence as to the constitution of natural kynurenic acid, a substance which has some close connection with tryptophane in the metabolism of the dog.

In conclusion the author wishes to thank Dr. Hopkins of Cambridge for the interest which he has shown in the progress of this investigation.

## SOME OBSERVATIONS ON "EMOTIONAL GLYCOSURIA" IN MAN.

By OTTO FOLIN, W. DENIS AND W. G. SMILLIE.

(From the Laboratories of Biological Chemistry and Theory and Practice  
of Physic of Harvard Medical School, Boston.)

(Received for publication, March 20, 1914.)

Emotional glycosuria recently described by Cannon, Shohl and Wright<sup>1</sup> is an interesting phenomenon deserving further study. The fairly frequent occurrence of moderate glycosuria in the insane has long been known and since a large number of such patients are subject to intense emotional strain it would seem fairly plausible to assume in view of the results obtained by Cannon, Shohl and Wright on cats that many of the glycosurias occurring in the insane have a psychic origin. Mita<sup>2</sup> has recently reported the results of a large number of examinations on urines of insane persons and in this paper he suggests that there may be a certain relationship between glycosuria and the mental state independent of the more fundamental character of the mental disease. Mita found sugar in the urine to be particularly frequent in patients subject to fear and depressions.

In order to obtain additional data on the subject of glycosuria in the insane we have examined the urine of 192 patients at the McLean Hospital for the Insane. In twenty-two of these we found unmistakably positive sugar reactions with Nylander's, Benedict's and the phenylhydrazine tests.

The great majority, but not all of those who had sugar in the urine, suffered from depression, apprehension or excitement. Some of them had been in apparently the same condition with reference to the emotional state for several years. This is worth noting in connection with Cannon's suggestion that the immediate cause

<sup>1</sup>Amer. Journ. of Physiol., xxix, p. 280, 1912.

<sup>2</sup>For a review of the literature on the subject of glycosuria in the insane see Mita: *Monatsh. f. Psychiatrie u. Neurologie*, xxxii, p. 159, 1912.

of emotional glycosuria may be due to an increased secretion of epinephrine.

From Dr. Earl D. Bond we have received the report of 664 consecutive urine examinations made at the Danvers State Hospital for the Insane. These examinations were made immediately after admission of the patients to the hospital and would therefore presumably include a larger percentage of excited patients.

The number giving a positive test for sugar, fifty-eight, represents a smaller proportion of positive tests than we obtained on the McLean Hospital patients, the percentage being 8.7 as against our 12 per cent. This difference might, however, easily be due to differences of method in testing for the sugar. At all events it is clear that the percentage of the insane having fairly large traces of sugar (or more) in the urine is very large and if there is such a thing as emotional glycosuria in human beings, one would naturally expect to meet with it very frequently in this class of patients.

More definite evidence of the existence of emotional glycosuria in man is perhaps to be obtained by testing for sugar in the urine of students before and after important examinations. We have made a series of such tests on students in the Harvard Medical School as well as on students at Simmons College (for women). The following results were obtained:

Of thirty-four second-year medical students examined before and after an examination one had sugar both before and after the examination. Of the remaining thirty-three, six, or 18 per cent, had small but unmistakable traces of sugar in the urine passed immediately after the examination.

A similar study was made on second-year women students at Simmons College. Since these students were younger and presumably much more excitable than our medical students it was thought that even more striking results might be obtained. This expectation did not prove well founded. Out of thirty-six taking the examination and who had no sugar in the urine on the day before, six, or 17 per cent, eliminated sugar with the urine passed immediately after the examination.<sup>3</sup>

It seems reasonably certain from the results obtained that pronounced mental and emotional strain may produce temporary glycosuria in man.

<sup>3</sup>For the collection of these urines we are indebted to Dr. Alice F. Blood of Simmons College.

# STUDIES ON THE THEORY OF DIABETES. III.

## GLYCOLLIC ALDEHYDE IN PHLORHIZINIZED DOGS.

BY W. D. SANSUM AND R. T. WOODYATT.

(From the Otho S. A. Sprague Memorial Institute Laboratory of  
Clinical Research, Rush Medical College, Chicago.)

(Received for publication, March 21, 1914.)

Glycollic aldehyde (diose) has figured prominently in theories of carbohydrate metabolism by virtue of its known properties and because it represents the simplest sugar. There is little data obtained by direct biological experimentation with the substance itself. Paul Mayer<sup>1</sup> administered to rabbits an impure solution prepared by Wohl's method. The rabbits showed glycosuria and died. One of us<sup>2</sup> in 1910, fed a 20-gram dose of glycollic aldehyde made by the method of Fenton and Jackson to a case of diabetes mellitus of moderate severity. The diose did not appear in the urine and there was no significant rise in the urinary glucose or fall in the acetone body output. The bowels were loosened and there was some gastric discomfort so that larger doses were not used. Parnas and Baer<sup>3</sup> in 1912, perfused livers with a similar solution and reported an increase of glycogen, thus apparently showing that diose may form glucose in the body. In the same year Ida Smedley<sup>4</sup> found that liver emulsion had the power to destroy glycollic aldehyde. Barrenscheen also reports the formation of glycogen from glycollic aldehyde by liver tissue.<sup>5</sup>

In view of the fact that diose in the presence of weak alkali may be condensed to yield a mixture of higher sugars, the character of which may be varied with the conditions of the experiment; and of the data of Parnas and Baer, it would seem reasonably certain that

<sup>1</sup> *Zeitschr. f. physiol. Chem.*, xxxviii, p. 351, 1903

<sup>2</sup> *Journ. Amer. Med. Assoc.*, lv, p. 2109, 1910.

<sup>3</sup> *Biochem. Zeitschr.*, xli, p. 386, 1912.

<sup>4</sup> *Journ. of Physiol.*, xlv, p. 203, 1912

<sup>5</sup> *Biochem. Zeitschr.*, lviii, p. 300, December, 1913.

if given to a fully phlorhizinized dog it should prove capable of conversion into glucose like other sugars, although this has not been established. The present experiments were begun with the expectation that diose would pass readily over into glucose and appear as such in the urine. The material used was prepared by decomposing a known weight of the dihydroxy-fumaric anhydride of Fenton and Jackson. An apparent conversion was finally obtained, but in earlier experiments there was either a rise in the total output of sugar with an accompanying rise in the nitrogen so that G:N remained constant, or a rise of G:N coincident with a fall in the total secretion. It was not considered that either of these effects afforded final proof of a new glucose formation from the glycollic aldehyde. The former experiments were interpreted as signifying an increased protein breakdown coincident with oxidation of the diose, the latter as toxic effects superposed on those of the phlorhizin itself and entailing irregularities of the metabolism or the action of the kidneys. These confused results led to a more general study of the conditions which favor the quantitative excretion of sugars in general and some of their isomers such as lactic acid. In general it seemed that concentrated doses were inappropriate, whereas dilute and protracted doses favored conversion into glucose and excretion as such. The attempt had first been made to give glycollic aldehyde by stomach tube. One hundred cc. of a 10 per cent solution so given to a fully phlorhizinized dog were retained for half an hour, then vomited. Catharsis also followed. A 1 per cent solution having almost no taste was drunk voluntarily by dogs. Twenty-five to fifty cc. of this were given every twenty minutes but after the reception of a quantity corresponding to 1 to 2 grams of glycollic aldehyde, more was refused and emesis occurred. Having found it difficult to have the material retained when given *per os* to the fully phlorhizinized dogs, the subcutaneous method—and once the intravenous—was thereafter employed. At first 6.6 grams of glycollic aldehyde in 75 cc. of water were injected subcutaneously all at one time. The absolute quantity of sugar in the urine rose while G:N remained practically constant. As the absolute quantity of reducing substance rose there was an actual diminution of the dextro rotation making a large discrepancy between the polariscopic and titration figures for the period. This would be most simply explained by an increase of the laevo-rotatory

hydroxy-butyric acid or of some compound of glycollic aldehyde with glucuronic acid. Diose itself was not demonstrated. The same phenomenon was found in five out of six experiments and is receiving further study. Six and four-tenths grams of diose in 100 cc. of water similarly given caused a rise of G:N, but following the dose the animal became decidedly weakened, the total secretion falling to a low level, so that the significance of the ratio was in doubt. One and four-tenths grams of diose in 3 per cent solution injected intravenously were followed by a slight rise of G:N and a slight fall in the total reducing and dextro-rotatory power of the urine. Seven and one-half grams of diose in 5 per cent solution were injected subcutaneously in three 50-cc. portions half an hour apart. In this experiment there was a rise in the absolute quantity of sugar excreted and in the nitrogen but in this case also a rise in G:N with a quantity of extra glucose reckoned as 1.8 grams. Three and seventeen-one-hundredths grams of diose in 1 per cent solution given subcutaneously 25 cc. at a time, every fifteen minutes until all was used (three and one-fourth hours) caused a rise in the absolute quantity of sugar excreted with a rise of G:N and a total quantity of extra sugar reckoned at 2.43 grams or 76.3 per cent of the given substance. 5.06 grams of diose in 400 cc. of water (1.2 per cent sol.) given subcutaneously in portions of 20 cc. every fifteen minutes (total time five hours) showed an amount of extra glucose equivalent to 45 per cent of the weight of glycollic aldehyde given. During the second 8-hour period after the injection, as observed also in several other experiments, there occurred a pronounced fall both of the nitrogen and sugar excretion.

The experiments fall into two groups: those in which there has been no evidence of a new formation of glucose, and those in which there has been a small quantity of "extra" sugar following the administration of the diose. Dakin<sup>6</sup> has called attention to the necessity of exercising caution in the interpretation of small increases of sugar as signifying an actual conversion into glucose of a given substance in experiments of this kind. We have also recently seen an increase of sugar following the administration of substances whose conversion into glucose is improbable. In such experiments the rise in sugar has sometimes been followed in later

<sup>6</sup> This *Journal*, xiv, p. 328, 1913.

periods by a corresponding fall—when the experiment has been long enough continued. Further experiments will be made with glycollic aldehyde. In the meantime judgment is reserved as to whether or not this substance is actually capable of conversion into glucose under the conditions of these experiments.

## EXPERIMENTS.

*Material.* The glycollic aldehyde used was prepared from the crystalline product described by Fenton and Jackson<sup>7</sup> as dihydroxy-maleic (later as dihydroxy-fumaric) anhydride. In the preparation use was made of the modification suggested by Nef.<sup>8</sup> The crystals were washed with ice water until pure white, then dissolved in absolute methyl alcohol from which they were reprecipitated by the addition of one and one-quarter volumes of ice water. The final product was lustrous, snow-white and when dissolved in water and boiled, left only a faint trace of acid in the solution. For the purposes of the animal experiments the quantity

PERIODS	GLUCOSE			N	G : N	REMARKS
	Titration	Polariscope	Difference			
I	6.90	6.13	0.77	1.50	4.60	6.6 gms. diose in 100 cc. water, subcut.; at one time. Insignificant rise in G:N with absolute increase of both glucose and nitrogen.
II	7.00	6.37	0.63	1.98	3.53	
III	6.50	5.15	1.35	1.87	3.53	
IV	7.50	4.59	2.91	2.06	3.65	
V	8.00	6.41	1.53	2.52	3.18	
I	6.12	6.03	0.09	2.15	2.85	1.4 gms. diose in 3 per cent sol., intraven., during 15 min. G:N shows slight rise for last two periods but no absolute rise in glucose.
II	5.50	5.25	0.25	2.02	2.72	
III	5.60	5.50	0.10	1.95	2.87	
IV	5.20	4.46	0.74	1.73	3.00	
V	4.61	4.26	0.35	1.45	3.17	
I	8.13	6.29	1.84	2.55	3.19	6.4 gms. diose in 100 cc. water subcut. Rise in G:N but fall in absolute glucose figures, etc.
II	6.88	5.13	1.75	1.97	3.48	
III	4.38	2.25	2.13	0.97	4.19	
IV	0.80	0.77	0.27	0.13	6.34	

<sup>7</sup> *Journ. of Chem. Soc.*, lxxv, p. 4.

<sup>8</sup> *Liebig's Annalen*, cccxxxv.

PERIODS	GLUCOSE			N	G : N	REMARKS
	Titra- tion	Polari- scope	Differ- ence			
I	11.25	10.23	1.02	2.97	3.78	7.5 gms. diose in 5 per cent sol., subcut., in 3 doses of 50 cc. at half hr. intervals. Using G:N = 3.72, extra glucose = 1.77 gms.
II	11.87	11.19	0.68	3.24	3.66	
III	13.75	12.43	1.32	3.43	4.00	
IV	16.25	14.28	1.97	4.16	3.90	
I	10.13	9.08	1.05	2.97	3.41	3.17 gms. diose as 317 cc. of 1 per cent sol., subcut., 25 cc. every 15 min. Using G:N = 3.65 extra glucose (in 8 hrs.) = 2.43 gms.
II	13.75	12.50	1.25	3.28	4.19	
III	10.62	10.47	0.15	2.73	3.88	
I	16.88	15.95	0.93	4.60	3.65	5.06 gms. diose as 400 cc. of 1.2 per cent sol., subcut., 20 cc. every 15 min. Using G:N = 3.60 extra glucose = 2.26 gms. (8 hrs.).
II	15.63	14.76	0.87	4.40	3.55	
III	16.88	15.35	1.53	4.06	4.15	
IV	13.75	12.61	1.14	3.33	4.12	
V	8.75	8.03	0.72	2.92	3.00	

of glycollic aldehyde was calculated as 45 per cent of the quantity of pure dry dihydroxy-fumaric anhydride added to 100 cc. of water and decomposed. Preliminary tests with the pure substance showed that the reducing power of the diose solution resulting from the decomposition of 1 gram of the anhydride was equivalent in terms of glucose to 68 per cent of the calculated quantity of diose (titration by Bang's method). For subcutaneous injection the slight acidity was neutralized just prior to injection by means of dry bicarbonate of sodium.

*Methods.* Dogs were phlorhizinized by the subcutaneous injection of 1 gram of phlorhizin rubbed up in 20 cc. of olive oil, every twelve hours. The periods were of eight hours each. Urine was removed by catheter. Glucose was determined by means of the polariscope and the titration method of Bang and Bohmannson, nitrogen by the Kjeldahl method. For the detection of unchanged glycollic aldehyde in the urine use was made of the fact that glycollic aldehyde reduces Fehling's solution and silvers the walls of test tubes containing ammoniacal silver oxide solutions in the cold. It is also volatile at 45° and 15-20 mm. pressure. The urine was accordingly distilled under these conditions and the distillate

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tested for aldehyde by means of Schiff's reagent as well as for any volatile reducing substance. The tests were always negative both in the native urine and in the distillate.

### SUMMARY.

Glycollic aldehyde has been prepared from the crystalline dihydroxy-fumaric anhydride of Fenton and Jackson. Given to fully phlorhizinized dogs in single doses of about 5 per cent strength subcutaneously it has apparently caused an increased breakdown of protein as evidenced by a rise in the urinary nitrogen and a corresponding increase of the sugar while G:N remained constant. The initial rise has been followed in later periods by a fall in all the urinary components examined. When given very slowly in dilute 1 per cent solution there has been observed an absolute rise in the glucose excretion out of proportion to the rise in the nitrogen, hence an increased G:N ratio not due merely to a lessened nitrogen output but suggestive of a new formation of glucose out of glycollic aldehyde itself. Some 45 to 75 per cent of the glycollic aldehyde given appears to have escaped oxidation and become converted into glucose in these latter experiments.

Judgment is reserved as to whether the small increases of reducing substances seen in these experiments are due to a conversion of glycollic aldehyde into *d*-glucose or to some other process.

## THE VELOCITY OF HIPPURIC ACID FORMATION AND ELIMINATION FROM THE ANIMAL BODY.

By A. M. RAIZISS, G. W. RAIZISS AND A. I. RINGER.

(From the John Herr Musser Department of Research Medicine, and from the Department of Physiological Chemistry of the University of Pennsylvania, Philadelphia, Pa.)

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Different workers in recent years have shown that a great deal more glycocoll is available for the formation of hippuric acid than is found preformed in the proteins of the body. Magnus-Levy<sup>1</sup> found in his experiments on rabbits that 28 per cent of the total nitrogen could consist of glycocoll nitrogen. One of us<sup>2</sup> found that on giving 25.4 grams of benzoic acid to a goat weighing about 40 kgm. an amount of glycocoll was eliminated in the hippuric acid which corresponded to 38.4 per cent of the total nitrogen in the urine.

Wiechowski,<sup>3</sup> in an exhaustive study of the laws of the formation of hippuric acid, came to the conclusion that as much as 64 per cent of the total nitrogen can be eliminated in the form of glycocoll, and that the higher amino-acids in their catabolism pass through a glycocoll stage before they are finally broken down to urea. He came to that conclusion by assuming that the hippuric acid which is excreted in twenty-four to thirty-six hours is synthesized during the first eight hours after the administration of benzoic acid and that all the hippuric acid nitrogen is derived entirely from the protein that is metabolized during the first eight hours.

In these experiments it was our object to determine the velocity of formation of hippuric acid and also the velocity of its elimination in the urine, with the object of testing the correctness of Wiechowski's conclusions.

<sup>1</sup> Magnus-Levy: *Biochem. Zeitschr.*, vi, p. 523, 1907.

<sup>2</sup> Ringer: this *Journal*, x, p. 327, 1911.

<sup>3</sup> Wiechowski: *Beitr. z. chem. Path. u. Physiol.*, vii, p. 204, 1906.

*Methods.* Male rabbits were employed in all the experiments. They were kept in cages suitable for the quantitative collection of urine, and were catheterized at the end of each period. The nitrogen was determined by Kjeldahl; urea by Benedict; free benzoic acid<sup>4</sup> and combined benzoic acid by the methods of Folin and Flanders.<sup>5</sup>

*Rabbit 2* was allowed to fast for three days. In period IV 1.44 grams ( $\frac{N}{100}$ ) of sodium benzoate were given *per os*. As is seen from the table, only one-half the combined benzoic acid (hippuric acid) was eliminated during the first seven hours. It was, however, all eliminated within twenty-four hours.

*Rabbit 3* was treated like the preceding one. In period IV, 2.88 grams ( $\frac{N}{50}$ ) of sodium benzoate were given *per os*. In this case the elimination of all the combined benzoic acid took more than twenty-four hours.

*Rabbit 4.* In this experiment it was our object to find out whether the slow elimination of the hippuric acid was due to slow synthesis or to slow excretion, as is maintained by Wiechowski. In period V, 2.01 grams ( $\frac{N}{100}$ ) of sodium hippurate, dissolved in 150 cc. of isotonic salt solution, were administered intravenously. The injection was made in one of the ear veins (without anesthesia) and lasted twenty minutes. *During the first nine hours practically all the hippuric acid was eliminated in the urine.*

These experiments justify the conclusion that hippuric acid is not eliminated with difficulty, and that the slow elimination of hippuric acid after benzoic acid administration is due either to slow resorption or to slow synthesis.

Wiechowski, in calculating the percentage relationship between hippuric acid nitrogen and total nitrogen, compared the former with only that fraction of the latter which was eliminated in the first eight hours. On the basis of this calculation he obtained the very high figure of 64 mentioned above. In the light of our present work this is not justified. The percentage relationship must be compared with the entire twenty-four hours' nitrogen, which reduces it to one-third, namely, 21, which is about the same as that observed by Magnus-Levy.

<sup>4</sup> Folin and Flanders: *Journ. Amer. Chem. Soc.*, xxxiii, p. 1622, 1911.

<sup>5</sup> Folin and Flanders: this *Journal*, xi, p. 257, 1912.

After the hippuric acid administration, a rise in the free benzoic acid elimination is noticeable, which is interesting as it may signify a reversibility in the reaction.

*Rabbit 2.*

PERIOD	WEIGHT	NO. OF HOURS	N	UREA N	FREE BENZOIC ACID	COMBINED BENZOIC ACID	REMARKS
I	1.84	24			0.014	0.012	{ 1.44 grams sodium benzoate = 1.22 grams benzoic acid, given <i>per os</i> .
II	1.78	24	0.778	0.709	0.015	0.012	
III	1.76	24	0.945	0.855	0.016	0.013	
IV A	1.66	7.3	0.495	0.351	0.024	0.621	
IV B		16.7	0.604	0.503	0.040	0.610	
V	1.53	24	1.80		0.010	0.028	
VI	1.48	24	2.42		0.018	0.011	
VII		24	2.60		0.014	0.015	

*Rabbit 3.*

I	1.62	24	0.84	0.76			{ 2.88 grams sodium benzoate = 2.44 grams benzoic acid, given <i>per os</i> .
II	1.61	24	0.73	0.70			
III	1.54	24	0.76	0.72	0.016	0.047	
IV A		12	0.77	0.58	0.124	0.898	
IV B		12	0.65	0.51	0.022	0.794	
V		24	1.25	1.05	0.015	0.438	
VI	1.23	24	1.29	1.18			

*Rabbit 4.*

IV	1.88	24	0.697	0.626	0.016	0.031	{ 2.01 grams sodium hippurate dissolved in 150 cc. isotonic salt solution, given intravenously.
V A		9	0.401	0.241	0.052	1.061	
V B	1.85	10	0.254	0.214	0.009	0.034	
VI					0.011	0.027	



# ON REDUCTION OF AMMONIUM MOLYBDATE IN ACID SOLUTION.

BY C. W. MILLER AND A. E. TAYLOR.

(From the Department of Physiological Chemistry, University of Pennsylvania, Philadelphia, Pa.)

(Received for publication, March 24, 1914.)

Ammonium molybdate, in acid solution, is reduced to an oxide stage, with the production of a blue color, by many substances of biological importance. Our attention was first attracted to this subject through an accidental observation. We were engaged in the attempt to work out a technique for the nephelometric estimation of phosphoric acid, using a heavy solution of cane sugar for the medium of suspension. When the mixture containing acid, ammonium molybdate and cane sugar was heated, a deep blue color was rapidly produced. The same reaction was slowly accomplished in the cold. Further study indicated that this reaction was due to the levulose split off from the cane sugar by the acidity of the solution. Later we found that this reaction had been described as a test for levulose by Pinoff.<sup>1</sup> Later study of the reaction has yielded instructive results. The reaction is in some ways a rather specific one, since comparatively few reducing substances, otherwise active, are able to effect in acid solution the transfer of the metal to the stage of blue oxide. A review of the literature dealing with this metal indicates firstly that more than one oxide stage presents a blue color, and secondly that the several oxide stages are so unstable and interchangeable that a qualitative relation of a particular reagent to a particular oxide is not now demonstrable.

The solution we have most commonly used consists of 30 grams of ammonium molybdate and 25 cc. of sulphuric acid dissolved in 1 liter of distilled water. The test is made by adding to a few cubic centimeters of the reagent about an equal volume of the

<sup>1</sup> Pinoff: *Ber. d. d. chem. Gesellsch.*, xxxviii, p. 3308.

solution of the substance to be tested (it must be free of phosphoric acid) and standing the test tube in a water bath for from ten minutes to a half hour. A deep blue, somewhat lighter in color (*i.e.*, with less of the purple tinge) than the blue of copper sulphate in alkaline solution, constitutes the reaction. On dilution, the color fades to a greenish blue. On standing the color is apt to fade; sometimes it changes to a pale purple.

Certain substances yield a positive reaction in a most striking manner, using mere traces of the reducing substance. These are levulose (cane-sugar, raffinose, inulin), dioxy-acetone, hydrazines, indol and skatol, oxalic-acetic ester, furfural, and aceto-acetic ethyl ester. Many other substances yield the reaction in a distinct but not striking fashion, with the use of a much larger amount of the substance being tested. Included in this group are glucose, galactose, lactose, maltose, pyrogallol, tricresol and tryptophane. Metol (monomethyl-*p*-amino-*m*-cresol) and amidol (diaminophenol) react positively but atypically. Many substances that possess reducing properties in other directions do not yield this reaction. Negative are benzaldehyde, acetaldehyde, formaldehyde (the faintest trace of color is evolved on prolonged heating) acetone, methyl-propyl ketone, glycerol, chloroform, levulinic acid, uric acid, creatine, creatinine, acrolein, pyroracemic acid, phenol, *p*-nitrophenol, hydroquinone, salicylic acid, tyrosine, thymol, orcein, hydroxylamine, vanillin, phloroglucine, tannic acid, sulphide, sulphite and phenyl sulphate. Some substances that do not give the reaction in acid solution react positively in alkaline reaction; thus, phenol, tyrosine, thymol, orcein, tannic acid, and uric acid. Tricresol, which acts in acid solution, does not react in alkaline reaction.

On reviewing these substances, several interesting facts appear. Thus indol and skatol react strongly; the parent substance, tryptophane, reacts feebly. Plain phenol and tyrosine do not react. We are however inclined to believe that derivatives of tyrosine and phenylalanine contained in the urine do react, since the degree of reduction effected by urine seems to be more than can be accounted for by the indol and skatol present. The ketone group does not react—but the ketone sugar reacts very strongly. That the primary and secondary alcoholic groups, ketone and methyl, *per se* do not reduce, is seen in the negative findings with acetone and

glycerol. But when a primary alcoholic group is attached to a ketone group, as in levulose or dioxy-acetone, the reaction is pronounced. The aldehyde group does not react strongly, as evidenced by the faint reaction with the aldoses as well as with the simple aldehydes. Hydrazine reacts strongly, as sulphate or chloride; hydrazine reacts best in alkaline reaction, phenyl hydrazine, however, best in acid solution. The hydrazines act energetically in the cold. They not only reduce the molybdate from the ammonium salt, but also from the complex combination with phosphoric acid, upon which behavior we have founded a method for the colorimetric estimation of traces of phosphoric acid.

It is interesting to contrast these results with those reported by Folin<sup>2</sup> and Lewis<sup>3</sup> with the use of the tungsten and molybden reagents of Folin, used in alkaline reaction. The reduction in acid solution is much more selective and is effected by fewer substances. The Folin colorimetric method for the estimation of uric acid rests upon the fact that if the phenol and indol bodies are removed from the urine, uric acid alone remains to react with the Folin reagent, with the production of the blue color that is then measured by comparison with a standard. These benzene derivatives are removed by evaporating the urine to dryness in the presence of oxalic acid and extracting the residue with a mixture of ether and methyl alcohol. Now our reagent reacts with these benzene derivatives, but not with uric acid. When this reagent is applied to the extract and residue respectively of the Folin method, the extract is found to give a positive result and the residue gives no reaction—*i.e.*, the residue is free of the reacting benzene derivatives, thus proving the operation of the Folin procedure to be quantitative and reliable. The reaction of normal urine to our reagent is due, so far as we are able to determine, solely to derivatives of tryptophane, tyrosine and phenylalanine, most largely to indol and skatol.

Normal blood serum, freed of protein, does not give a demonstrable reaction, or at most the merest trace, comparable to that effected by a small amount of glucose. Upon the occasion of the last meeting of the Society of Biological Chemists, when Professor J. J. Abel of Johns Hopkins University demonstrated his method

<sup>2</sup>Folin and Denis: this *Journal*, xii, pp. 239, 245; xiii, p. 469; xiv, p. 95; Folin and Macallum: *ibid.*, xiii, p. 363.

<sup>3</sup>Lewis and Nicolet: *ibid.*, xvi, p. 369.

of so-called "vividiffusion," we were able to apply the test to the dialyzation fluid, which gave a reaction far more pronounced than blood serum, and much more marked than the reduction effected by a solution of glucose of the concentration present. From this it may be inferred that the named aromatic substances are not present in the blood except in the most minute traces, that they are promptly and almost completely eliminated—into the urine normally—into the diffusion fluid of the "vividiffusion" experiment.

The availability of this reagent for determining the presence of levulose in the urine depends upon the fact that the latter substance gives a far more striking reaction than either normal urine or glucose solutions. The reducing power of urine in the natural state cannot be studied satisfactorily because of the confusing effect of the reaction between the phosphates and the molybdate. The phosphates may be conveniently removed as follows. To 5 cc. of urine are added 10 cc. of a 4 per cent solution of lead acetate. After the mixture has been well agitated, 15 cc. of a 6 per cent solution of sodium sulphate are added, the whole well mixed and filtered. The filtrate is practically free of phosphates and lead. Five cc. of this filtrate are then mixed with 15 cc. of a 5 per cent solution of sulphuric acid and the solution thus obtained represents a dilution of the original urine of about 24:1. This considerable dilution is recommended because the colors are more easily compared when not too intense. The reducing power of normal urine may now be studied by mixing in a test tube one part of the final diluted solution with one part of the reagent and placing the tube in a boiling water bath for ten minutes. Normal urine yields first a faint green, becoming deeper, finally a moderate blue with a greenish tinge. If comparable results are desired, time and temperature must be held constant.

Now if we dilute the contents of this tube, containing the reaction of normal urine, about twenty-five times (a total dilution of the urine of about 600:1), the color will practically disappear. If however levulose be present, as in the clinical test for hepatic functionation, a green or blue color will persist to a much greater dilution. Proceeding as follows, a urine containing as little as 0.5 per cent of levulose will yield a positive reaction. One part of the mixture of filtrate and 5 per cent sulphuric acid, described above, is diluted with 25 parts of distilled water; and one part of

this mixture is added to an equal volume of the reagent and tested and heated, as described, in a water bath. Working with such high dilutions, the time in the bath may be extended to twenty or twenty-five minutes before normal urine will give more than a very faint coloration, while the levulose-containing urine yields a goodly blue. In making the test, a control with a normal urine should be run through with the test. A trace of glucose in the urine does not seriously interfere with the test for levulose, since glucose does not react as strongly as do the normal aromatic derivatives of the urine. To what extent this test is capable of clinical application, has not been determined. Instead of the above-described clarification with lead, the phosphates of the urine may be removed with barium hydrate or ferric chloride plus ammonia; in either case, the filtrate is practically free of phosphates.

We have finally compared the reduction due to the aromatic substances of the urine, extracted with ether-methyl-alcohol of Folin, with the colors developed in the oxidation tests for indican, using especially the bimolecular oxidation with isatine. We have not found the reactions to be parallel. On the one hand, the molybdate reagent reacts with other aromatic derivatives than indol and skatol; on the other hand, the oxidation and extraction of indol is rarely quantitative. It is however possible that our reagent, or the reagent of Folin, might be developed to serve for the colorimetric estimation of the indol and phenol derivatives contained in urine.



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